

INTESTINAL PERMEABILITY AS A MARKER OF GASTROINTESTINAL HEALTH

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ACADEMIC DISSERTATION

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*“This is how you do it:
You sit down at the keyboard and you
put one word after another until it is
done. It is that easy, and that hard.”*

-Neil Gaiman

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS	7
MAIN ABBREVIATIONS	8
ABSTRACT	9
<u>1 INTRODUCTION</u>	<u>11</u>
<u>2 REVIEW OF LITERATURE</u>	<u>12</u>
2.1 INTESTINAL BARRIER FUNCTION	12
2.1.1 COMPONENTS OF THE INTESTINAL BARRIER	12
2.1.2 PATHWAYS OF PERMEABILITY	14
2.1.3 TIGHT JUNCTIONS	16
2.1.4 INTESTINAL PERMEABILITY AS A MEASURE OF FUNCTIONALITY	20
2.2 FACTORS THAT AFFECT INTESTINAL PERMEABILITY	22
2.2.1 INTESTINAL INFLAMMATION	22
2.2.2 INTESTINAL MICROBES	23
2.2.3 DIETARY FACTORS	25
2.2.4 PSYCHOLOGICAL AND PHYSICAL STRESS	28
2.2.5 CHEMOTHERAPY	30
2.3 HOW TO MEASURE INTESTINAL PERMEABILITY?	33
2.3.1 DIRECT PERMEABILITY ASSAYS IN VITRO	33
2.3.2 THE USSING CHAMBER FOR EX VIVO PERMEABILITY ASSAYS	33
2.3.3 DIRECT PERMEABILITY ASSAYS IN VIVO	34
2.3.4 INDIRECT MEASURES OF INTESTINAL BARRIER FUNCTION	39
<u>3 AIMS OF THE STUDY</u>	<u>42</u>
<u>4 MATERIALS AND METHODS</u>	<u>43</u>
4.1 EXPERIMENTAL ANIMALS AND STUDY SUBJECTS	43
4.1.1 EXPERIMENTAL ANIMALS	43
4.1.2 STUDY SUBJECTS	43
4.2 STUDY DESIGNS	44
4.2.1 STUDY I/II	44
4.2.2 STUDY III	45
4.2.3 STUDY IV	45
4.3 SAMPLE COLLECTION AND ANALYSES	47
4.3.1 MEASUREMENT OF IN VIVO INTESTINAL PERMEABILITY	47
4.3.2 TISSUE PREPARATION FOR THE USSING CHAMBER	47
4.3.3 MEASUREMENT OF IN VITRO INTESTINAL PERMEABILITY IN THE USSING CHAMBER	48
4.3.4 MEASUREMENT OF ELECTROPHYSIOLOGICAL PARAMETERS IN THE USSING CHAMBER	49
4.3.5 TISSUE COLLECTION FOR HISTOLOGICAL ANALYSIS	49

4.3.6	HISTOLOGICAL ANALYSIS	49
4.3.7	IMMUNOFLUORESCENCE ANALYSIS FOR ENTERIC NERVES	50
4.3.8	GLOBAL METABOLOME ANALYSIS OF SERUM AND URINE	51
4.3.9	FECAL MICROBIOTA ANALYSIS	51
4.3.10	BIOCHEMICAL ANALYSES	52
4.3.11	ASSESSMENT OF CLINICAL SIGNS OF GASTROINTESTINAL DISTRESS	53
4.3.12	DATA ANALYSES	53
5	RESULTS	56
5.1	CLINICAL SIGNS OF GASTROINTESTINAL DISTRESS	56
5.1.1	CHEMOTHERAPEUTICS INDUCE BODY WEIGHT LOSS AND DIARRHEA	56
5.1.2	GASTROINTESTINAL SYMPTOMS DURING AND AFTER A 90-MIN RUNNING TEST	58
5.2	HISTOLOGICAL ANALYSES OF INTESTINAL TISSUES	59
5.2.1	CHEMOTHERAPEUTICS DISRUPT INTESTINAL MORPHOLOGY	59
5.3	MEASUREMENTS OF INTESTINAL PERMEABILITY	61
5.3.1	CHEMOTHERAPEUTICS INCREASE IN VIVO INTESTINAL PERMEABILITY TO IOHEXOL	61
5.3.2	RUNNING INCREASES IN VIVO INTESTINAL PERMEABILITY TO IOHEXOL	63
5.3.3	DCA INCREASES EX VIVO PERMEABILITY TO FLUORESCEIN IN MUCOSAL SEGMENTS	64
5.4	ANALYSIS OF FECAL MICROBIOTA	66
5.4.1	IRINOTECAN MODULATES THE COMPOSITION OF FECAL MICROBIOTA	66
5.5	ANALYSIS OF GLOBAL METABOLOME	68
5.5.1	CHEMOTHERAPEUTICS CHANGE THE GLOBAL METABOLIC PROFILE OF SERUM AND URINE	68
5.5.2	CHEMOTHERAPEUTICS INCREASE SERUM LEVELS OF FATTY ACID AND $N(CH_3)_3$ MOIETIES	70
5.5.3	CHEMOTHERAPEUTICS DECREASE URINARY HIPPURATE EXCRETION	70
5.6	BIOCHEMICAL ANALYSES	71
5.6.1	RUNNING INCREASES SERUM CONCENTRATIONS OF I-FABP	71
5.6.2	OTHER BIOCHEMICAL MEASUREMENTS	71
6	DISCUSSION	74
6.1	CHEMOTHERAPY-INDUCED GASTROINTESTINAL TOXICITY	74
6.1.1	EXPERIMENTAL MODELS AND STUDY SETTING	74
6.1.2	DOES IOHEXOL PERMEABILITY REFLECT THE SEVERITY OF CIGT?	76
6.1.3	WHAT DO THE MICROBIAL AND METABOLIC CHANGES REVEAL ABOUT THE MECHANISMS OF CIGT?	76
6.2	RUNNING-INDUCED GASTROINTESTINAL DISTRESS	79
6.2.1	DOES INTESTINAL PERMEABILITY EXPLAIN GASTROINTESTINAL SYMPTOMS DURING RUNNING?	79
6.2.2	IS INTESTINAL ISCHEMIA THE CAUSE OF INCREASED INTESTINAL PERMEABILITY AFTER RUNNING?	80
6.2.3	DOES SERUM LPS ACTIVITY CONTRIBUTE TO GASTROINTESTINAL SYMPTOMS DURING EXERCISE?	80
6.3	DCA-INDUCED INCREASE IN MACROMOLECULAR PERMEABILITY	82
6.3.1	DOES DCA INCREASE MACROMOLECULAR PERMEABILITY VIA ACTIVATION OF ENTERIC NERVES?	82
7	CONCLUSIONS	84

ACKNOWLEDGEMENTS	85
REFERENCES	88
ORIGINAL PUBLICATIONS	112

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (Studies I-IV):

- I. Forsgård R.A., Korpela R., Holma R., Lindén J., Frias R., Spillmann T., Österlund P. Intestinal permeability to iohexol as an in vivo marker of chemotherapy-induced gastrointestinal toxicity in Sprague-Dawley rats. *Cancer Chemotherapy and Pharmacology*. 2016; 78:863-874.
- II. Forsgård R.A., Marrachelli V.G., Korpela K., Frias R., Collado M.C., Korpela R., Monleon D., Spillmann T., Österlund P. Chemotherapy-induced gastrointestinal toxicity is associated with changes in serum and urine metabolome and fecal microbiota in male Sprague-Dawley rats. *Cancer Chemotherapy and Pharmacology*. 2017; 80:317-332.
- III. Karhu E.*, Forsgård R.A.*, Alanko L., Alfthan H., Pussinen P., Hämäläinen E., Korpela R. Exercise and gastrointestinal symptoms: Running-induced changes in intestinal permeability and markers of gastrointestinal function in asymptomatic and symptomatic runners. *European Journal of Applied Physiology*. 2017; 117:2519-2526.
- IV. Forsgård R.A., Korpela R., Stenman L.K., Österlund P., Holma R. Deoxycholic acid induced changes in electrophysiological parameters and macromolecular permeability in murine small intestine with and without functional enteric nervous system plexuses. *Neurogastroenterology and Motility*. 2014; 26:1179-1187.

*Equal contribution

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MAIN ABBREVIATIONS

¹ H-NMR	1-mm proton nuclear magnetic resonance
5-FU	5-fluorouracil
ALD	Alcoholic liver disease
CD	Crohn's disease
CIGT	Chemotherapy-induced gastrointestinal toxicity
⁵¹ Cr-EDTA	⁵¹ Cr-labeled ethylenediaminetetraacetic acid
DCA	Deoxycholic acid
ELISA	Enzyme-linked immunosorbent assay
FABP	Fatty acid-binding protein
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
JAM	Junctional adhesion molecule
LPS	Lipopolysaccharide
MLCK	Myosin light-chain kinase
NAFLD	Non-alcoholic fatty liver disease
NOD ₂	Nucleotide-binding oligomerization domain-containing protein-2
Papp	Apparent permeability coefficient
PCA	Principal component analysis
PEG	Polyethylene glycol
PLS-DA	Partial least squares-discriminant analysis
PRR	Pattern-recognition receptor
PUFA	Polyunsaturated fatty acids
T ₁ D	Type-1-diabetes
T ₂ D	Type-2-diabetes
TER	Transepithelial resistance
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
TTX	Tetrodotoxin
UC	Ulcerative colitis
ZO	Zonula occludens

ABSTRACT

Intestinal permeability is a key measure of gastrointestinal function and studies have associated increased intestinal permeability with several different pathologies. Clinically, increased intestinal permeability could contribute to disease pathophysiology by allowing unwanted substances to enter circulation and stimulate inflammatory processes. Thus, a better understanding of the factors and mechanisms that affect intestinal permeability could offer new insights on how to manage these diseases and disorders.

The aim of this thesis was to investigate intestinal permeability changes under three separate settings. Chemotherapy-induced gastrointestinal toxicity is a major complication of cancer treatment and we examined if measuring intestinal permeability to iohexol could be used to assess the severity of this complication. We also hypothesized that chemotherapeutics affect the composition of intestinal microbiota and the global metabolome and studied how these alterations relate to increased intestinal permeability. In another study setting, we investigated whether intestinal permeability changes explain individual's susceptibility to gastrointestinal symptoms during exercise. Finally, we treated intestinal tissue segments with a deoxycholic acid (DCA) concentration associated with high-fat feeding and examined the mechanisms by which it affects macromolecular permeability.

Our results show that commonly used chemotherapeutics, running at a challenging pace for 90 min, and DCA concentration associated with high-fat feeding increase intestinal permeability. The observed increase in intestinal permeability after chemotherapy also correlated with the severity of gastrointestinal toxicity. Chemotherapy-induced gastrointestinal toxicity was also associated with unfavorable changes in the composition of intestinal microbiota which may play a role in the pathophysiology of intestinal complications during chemotherapy. Running-induced increase in intestinal permeability seems to result from intestinal ischemia but it did not explain the occurrence of gastrointestinal symptoms during the running test. The DCA-

ABSTRACT

induced increase in macromolecular permeability appears to stem from direct interaction with the mucosa. However, we also observed evidence that DCA affects the intestine via a neural mechanism. Overall, these results demonstrate how different stressors can impair intestinal barrier function and possibly lead to various symptoms and complications.

1 INTRODUCTION

The gastrointestinal tract forms one of the largest epithelial barriers between the outside world and the host. The intestinal epithelium acts as a gate-keeper that must grant passage to nutrients while limiting the flux of noxious substances. The proper function of this double duty requires several mechanisms that together uphold intestinal homeostasis.

Intestinal permeability is one of key measures of intestinal homeostasis and alterations in intestinal permeability have been associated with multiple pathologies, such as inflammatory bowel diseases (IBD) (Michielan and D'Incà, 2015), irritable bowel syndrome (IBS) (Camilleri et al., 2012), different autoimmune diseases (Bischoff et al., 2014; Vaarala, 2013), obesity, and obesity-related diseases (Teixeira et al., 2012a). These observations have led to investigations about the factors and mechanisms that affect intestinal permeability. In addition, studies have tried to elucidate the causality between increased intestinal permeability and the pathophysiology of these diseases. Overall, these studies have revealed that abnormal intestinal permeability is a fundamental feature of disrupted intestinal homeostasis and measuring intestinal permeability could provide a useful tool for assessing changes in gastrointestinal function.

In this thesis, we investigated changes in intestinal permeability in three different study setting. First, we examined how chemotherapeutics affect intestinal permeability and whether intestinal permeability changes reflect the severity of gastrointestinal toxicity observed after chemotherapy. We also studied the associations between intestinal permeability and metabolic and microbial changes following the administration of chemotherapeutics. Next, we probed the connection between intestinal permeability and gastrointestinal symptoms during exercise. Finally, we investigated the mechanisms by which deoxycholic acid (DCA) affects the intestinal barrier.

2 REVIEW OF LITERATURE

2.1 Intestinal barrier function

2.1.1 Components of the intestinal barrier

The intestinal epithelium forms a dynamic barrier between the outside environment and the host. This barrier has a challenging task of absorbing the necessary nutrients while simultaneously prohibiting the passage of harmful substances. The latter purpose is usually referred to as intestinal barrier function and the intestine has multiple mechanisms in place to achieve this function (for review, see Martini et al., 2017). The mechanisms of intestinal barrier function are summarized in Figure 1.

Firstly, the rapid turnover of intestinal cells requires that the epithelium must constantly be replenished by intestinal stem cells. The continuous differentiation of intestinal stem cells into intestinal epithelial cells is vital for normal intestinal homeostasis and for sustaining intestinal integrity. The intestinal stem cells can differentiate into four types of cells: goblet cells, Paneth cells, enteroendocrine cells or enterocytes. Each of these cell types plays a role in maintaining the intestinal barrier function (for review, see Goll and Granlund, 2015).

The first line of defense consists of secretory products. Epithelial goblet cells secrete mucins that form a thick, viscous layer of mucus which prevents pathogens from directly contacting the epithelia (Frey et al., 1996). Paneth cells produce antimicrobial agents such as defensins that help to clear pathogens from the epithelial environment (Goll and Granlund, 2015). Additionally, enteroendocrine cells secrete serotonin and other immunomodulatory neuropeptides (Worthington, 2015). Serotonin also regulates intestinal motility and secretion, events that flush away potentially noxious substances from the intestinal lumen.

REVIEW OF LITERATURE

The most important intestinal defense mechanism is the physical barrier comprising of intestinal enterocytes and of the junctional complexes that link adjacent enterocytes together (Turner, 2009). The junctional complex consists of desmosomes, adherens junction, and tight junctions. Desmosomes and adherens junctions provide the mechanical linkage between enterocytes whereas tight junctions seal the intercellular space and regulate solute flux through the paracellular pathway (for review, see Groschwitz and Hogan, 2009). This unique function makes tight junctions pivotal for maintaining the mucosal integrity and changes or defects in their composition compromises the intestinal barrier function (Marchiando et al., 2010a).

Underneath the epithelial layer resides the gut-associated lymphoid tissue (GALT) which provides the last line defense against invading pathogens. Microfold cells (M-cells) are specialized epithelial cells that uptake luminal antigens and present them to submucosal dendritic cells (for review, see Ohno, 2016). Dendritic cells can also sample antigens straight from the lumen by extending their dendrites through the intercellular space between enterocytes. Dendritic cells present the collected antigens further to T-cells and B-cells inducing their activation and differentiation (Ohno, 2016). The B-cells differentiate to plasma cells that secrete immunoglobulin A (sIgA) into the intestinal lumen. sIgA is the most abundant intestinal antibody and it effectively blocks pathogens from binding to the epithelium (Mantis et al., 2011).

In addition to the body's own mechanisms, the intestinal lumen harbors a naturally occurring ecosystem of numerous microbial species. These microbes consume nutrients, compete for living space, and secrete antimicrobial substances. All these mechanisms prevent the colonization of pathogenic microbes and thus contribute to the intestinal barrier function (for review, see Yu et al., 2012).

REVIEW OF LITERATURE

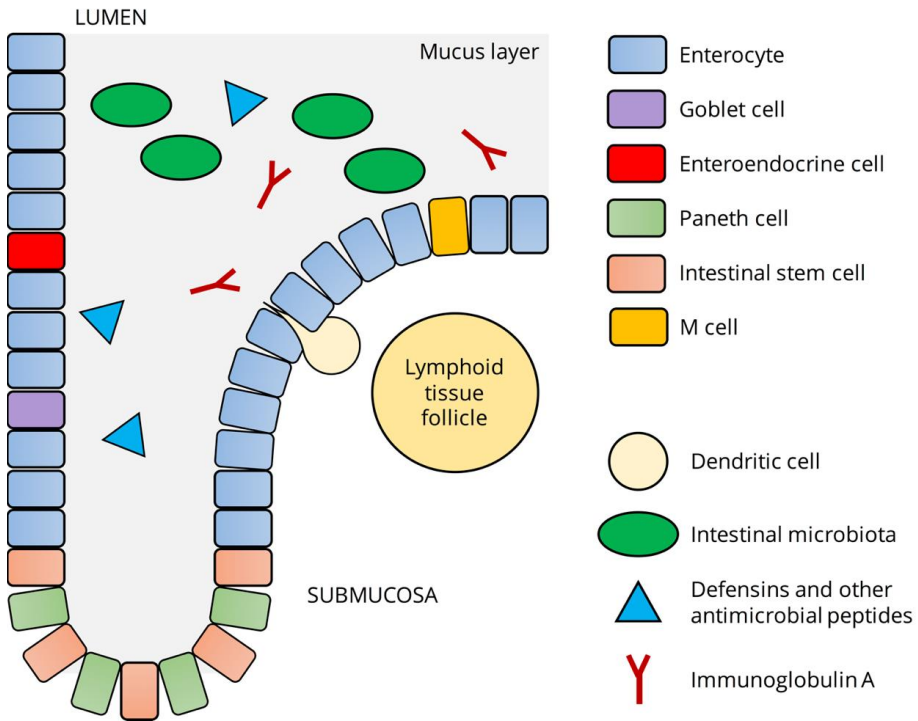


Figure 1. Schematic illustration of the different cell types in the intestinal epithelium and of the several mechanisms that contribute to intestinal barrier function (modified from Martini et al., 2017; and Ohno, 2016)

2.1.2 Pathways of permeability

Transepithelial passage of solutes across the intestinal epithelium occurs via two possible routes: the transcellular pathway and the paracellular pathway (Figure 2). The transcellular pathway refers to the energy-dependent and specific carrier-facilitated process of transporting nutrients, electrolytes, and other molecules through the cell membranes. In contrast, paracellular permeation happens between the epithelial cells and it is driven by passive movement of solutes against an electrochemical or concentration gradient (Groschwitz and Hogan, 2009).

Physically, epithelial permeability can be defined by the epithelium's ability to resist electric current. This measure is called transepithelial resistance (TER) and the total TER value of an epithelium is the sum of transcellular and paracellular resistance (Figure 2). Considering that epithelial cell membranes have high resistances that remain relatively constant across body's different epithelia, the paracellular resistance determines the overall TER value of given epithelium (Anderson and Van Itallie, 2009). Theoretically, TER values are inversely related to epithelial permeability: low TER values indicate a more permeable epithelium and vice versa. Functionally, the relevance of epithelial TER values connects to the purpose of individual epithelium. The intestinal epithelia are absorptive and secretory epithelia and thus have relatively low TER values (Anderson and Van Itallie, 2009) and subsequently high paracellular permeability. This makes paracellular permeability the main determinant of intestinal permeability (Turner, 2009) and the term intestinal permeability usually applies exclusively to paracellular permeability.

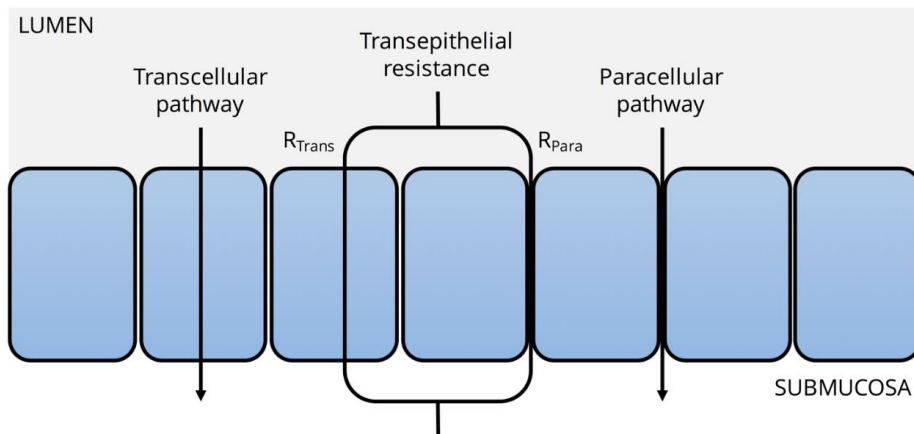


Figure 2. Solutes can cross the intestinal epithelium either transcellularly or paracellularly. Transepithelial resistance refers to the epithelium's ability to resist electric current and it is the sum of transcellular (R_{Trans}) and paracellular (R_{Para}) resistance (modified from Anderson and Van Itallie, 2009)

REVIEW OF LITERATURE

More detailed analyses of the paracellular pathway have revealed two distinct courses for paracellular permeation: the pore pathway and the leak pathway (for review, see Shen et al., 2011). The pore pathway selectively permits the passage of molecules based on their charge and size with molecules larger than 10 Å not allowed to pass (Anderson and Van Itallie, 2009; France and Turner, 2017). The leak pathway has a lower capacity than the pore pathway and exhibits no charge-selectivity but it grants translocation to molecules with sizes up to 125 Å (France and Turner, 2017). Evidence suggests that these two pathways are spatially arranged along the crypt-villus axis so that paracellular permeability increases towards the crypt (Fihn et al., 2000; Shen et al., 2011). Physiologically, this configuration makes sense because it would allow the flux of ions and small molecules at the villous tips on the epithelial surface (i.e. the pore pathway) and restrict the paracellular passage of larger molecules to the crypts far from luminal contents (i.e. the leak pathway). Additionally, the permeability of pore and leak pathway can be regulated independently (Anderson and Van Itallie, 2009; Shen et al., 2011) but it is not entirely clear how the interplay between these two pathways contributes to overall intestinal permeability.

2.1.3 Tight junctions

Tight junction is the most apical junctional complex connecting adjacent epithelial cells and it controls the paracellular flow of solutes through the intestinal epithelia. This makes tight junctions key moderators of not just intestinal permeability but also of the whole transepithelial transport (Turner, 2009).

Tight junctions are dynamic networks of over 50 different proteins with specific adaptor proteins connecting the actin cytoskeleton to the cell-membrane spanning proteins of the junctional complex (Ulluwishewa et al., 2011). The transmembrane part of the junctional complex includes five families of proteins: occludin, claudins, junctional adhesive molecules (JAMs), coxsackie virus and adenovirus receptor (CAR), and tricellulin. JAMs and CAR span the cell membrane only once whereas occludin, claudins, and tricellulin are tetra-

REVIEW OF LITERATURE

span proteins creating four transmembrane and two intracellular domains (Ulluwishewa et al., 2011). These intracellular domains anchor the transmembrane proteins to the cell's actomyosin ring via cytosolic scaffold proteins, such as zonula occludens (ZO) proteins (Figure 3) (Suzuki, 2013). How these individual components and their interactions affect the function of tight junctions is briefly summarized below.

Occludin was the first identified transmembrane tight junctional protein (Furuse et al., 1993) but initial reports showed that occludin knockdown does not have detrimental effects to the intestinal barrier function (Saitou et al., 1998; Schulzke et al., 2005). However, subsequent studies have revealed that silencing occludin expression in intestinal epithelial cells leads to increased paracellular flux of macromolecules (Al-Sadi et al., 2011a; Buschmann et al., 2013). These results suggest that occludin may mainly regulate the leak pathway with limited effects on the pore pathway. Silenced occludin expression also modifies the expression of other tight junctional proteins, most notably claudins (Al-Sadi et al., 2011a; Buschmann et al., 2013). Additionally, occludin phosphorylation controls the interaction between occludin and ZO-1 and thus can affect the properties of tight junctions (Groschwitz and Hogan, 2009).

REVIEW OF LITERATURE

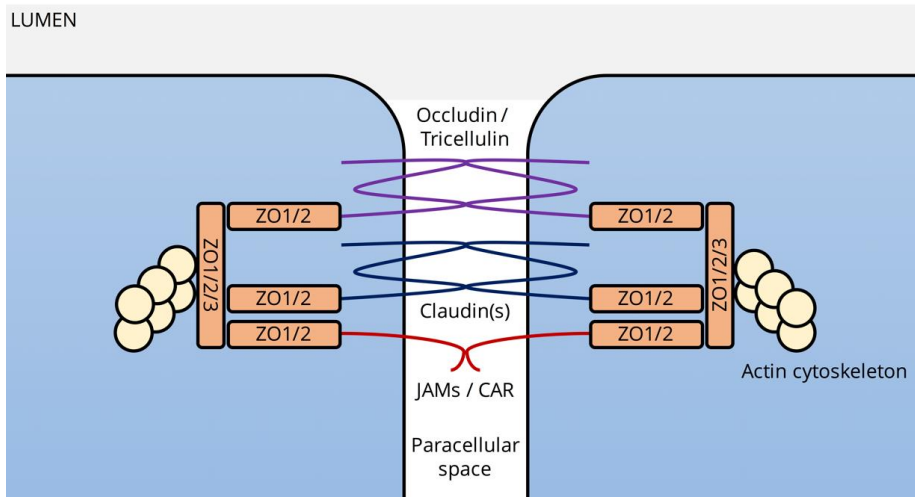


Figure 3. Schematic illustration of the tight junction connecting two enterocytes together. Transmembrane proteins occludin, claudins, junctional adhesive molecules (JAMs), and coxsackie virus and adenovirus receptor (CAR) interact with cytosolic zonula occludens -proteins 1-3 (ZO1/2/3) that anchor the junctional complex to the actin cytoskeleton. Tricellulin forms linkage between three adjacent enterocytes and localizes to tricellular junctions (modified from Ulluwishewa et al., 2011).

Claudins are a multiprotein family with humans expressing at least 26 different claudin isoforms (for review, see Garcia-Hernandez et al., 2017). These isoforms display different permeability properties which has led to the categorization of claudins as either barrier-enhancing or pore-forming. Pore-forming claudins, such as claudin-2 and claudin-10, create charge-selective channels that control the paracellular flux of ions and water (Anderson and Van Itallie, 2009). In contrast, barrier-enhancing claudins make tight junctions less permeable to certain ions. The fact that different claudin isoforms demonstrate charge-specificity has raised the hypothesis that claudins are important regulators of the pore pathway (Anderson and Van Itallie, 2009). Indeed, studies on transgenic mice have revealed the vital role claudins play in regulating epithelial barrier homeostasis (Garcia-Hernandez et al., 2017). For example, claudin-1^{-/-} mice die at birth due to skin barrier defects (Furuse et al., 2002) and

claudin-7^{-/-} mice exhibit mucosal ulcerations and intercellular gaps in the intestine (Ding et al., 2012). Also, intestinal claudin-7 deletion results in increased colonic permeability to small solutes (Tanaka et al., 2015). Interestingly, in humans the expression of claudin isoforms reflects their permeability properties. In the intestinal tract, the expression of barrier-enhancing claudins increases along the proximal-distal axis (Garcia-Hernandez et al., 2017; Lameris et al., 2013) which makes sense considering the distinct functions of small and large intestine (Martini et al., 2017). However, although the simple divide to barrier-enhancing and pore-forming claudins provides a useful framework to assess the function of individual claudins, the properties of tight junctions are always a combination of different claudin isoforms and their interactions (Garcia-Hernandez et al., 2017). Additionally, epithelial claudin expression, translocation, and modification is under constant regulation which further affects the permeability of tight junctions (Garcia-Hernandez et al., 2017).

JAMs and CAR are part of a larger family of proteins belonging in the immunoglobulin superfamily and they have a single transmembrane spanning domain. In addition to CAR, the intestinal epithelia express two JAM isoforms: JAM-A and JAM-4 (Suzuki, 2013). Overall, our understanding of their role in tight junctional regulation is lacking. JAM-A KO mice exhibit increased intestinal permeability and enhanced susceptibility to chemically-induced colitis compared to wild-type litter mates (Laukoetter et al., 2007). On the other hand, inactivation of the *Car* gene causes multiple phenotypes in adult mice but does not compromise the structure of intestinal tight junctions (Pazirandeh et al., 2011).

Tricellulin localizes in tight junctions between three enterocytes and it seems to regulate macromolecular flux through the tricellular junctions (France and Turner, 2017). However, the regulation of tricellular junctions in the context of intestinal permeability warrants more research.

REVIEW OF LITERATURE

ZO protein family of three proteins, ZO-1, ZO-2, and ZO-3, comprises of cytosolic scaffolding proteins that connect the transmembrane tight junction proteins to the actin cytoskeleton. ZO-1 and ZO-2 are crucial for normal development as ZO-1^{-/-} and ZO-2^{-/-} mice die already at embryonic state (France and Turner, 2017). In vitro, simultaneous silencing of ZO-1 and ZO-2 expression results in complete lack of tight junctions and increased paracellular flux (Umeda et al., 2006). Based on this and other evidence, the current understanding is that ZO proteins are necessary for the correct assembly of tight junctions (Suzuki, 2013).

In addition to tight junctional proteins, the paracellular pathway is regulated by several intracellular signaling proteins. Intracellular proteins, such as myosin light chain kinase (MLCK), protein kinase C (PKC), mitogen-activated protein kinases (MAPK), and the Rho family of GTPases, facilitate signals between the transmembrane proteins and the actin cytoskeleton and thus regulate the properties of tight junctions (Ulluwishewa et al., 2011). The MLCK pathway is perhaps the best characterized example of intracellular regulation of tight junctional permeability with multiple studies reporting increased paracellular permeability via MLCK-mediated actin cytoskeleton reorganization (Shen, 2012).

2.1.4 Intestinal permeability as a measure of functionality

In the context of intestinal epithelium, it is appropriate to distinguish between *intestinal barrier function* and *intestinal permeability*, two terms which are frequently used interchangeably. Intestinal barrier function refers to the epithelium's task of prohibiting the entry of harmful substances from the gut lumen whereas intestinal permeability can be defined as a measurable feature of intestinal barrier function (Bischoff et al., 2014). In theory, defects in any of the elements forming the intestinal barrier may result in alterations in intestinal permeability. Considering the vast literature associating increased intestinal permeability and different diseases, information about the factors and

REVIEW OF LITERATURE

mechanisms that modulate the intestinal barrier is vital for our understanding of normal intestinal homeostasis and disease pathogenesis.

To summarize, multiple structures and mechanisms form the intestinal barrier and contribute to maintaining the intestinal barrier function. Intestinal permeability is the measure of this function and it is mainly the sum of paracellular permeability making tight junctions vital for upholding the intestinal barrier. Tight junctions are dynamic protein complexes that are constantly assembled, modified, and maintained when epithelial cells proliferate to form the intact epithelium.

REVIEW OF LITERATURE

2.2 Factors that affect intestinal permeability

2.2.1 Intestinal inflammation

Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α and interferon (IFN)- γ , disrupt the intestinal barrier with numerous studies detailing their effects of intestinal permeability (for review, see Suzuki, 2013). The current evidence suggests that, although increased enterocyte apoptosis and cell shedding may also play a role (Watson and Hughes, 2012), pro-inflammatory cytokines increase intestinal permeability primarily via mechanisms involving tight junctional modifications (Suzuki, 2013; Turner, 2009). These modifications include MLCK-mediated changes in tight junctional structure and alterations in the expression of tight junctional proteins (Al-Sadi et al., 2014, 2012, 2011b, Bruewer et al., 2005, 2003, Clayburgh et al., 2006, 2005; Marchiando et al., 2010b; Yang et al., 2013; Ye et al., 2006).

Clinically, inflammatory mediators likely contribute to barrier defects observed in several pathological conditions but the interplay between them and intestinal permeability is especially interesting for the development of IBD. IBDs, such as Crohn's disease (CD) and ulcerative colitis (UC), are characterized as chronic inflammation of the intestine which leads to ulceration, intestinal bleeding, and diarrhea. The treatment of IBD relies on alleviating the underlying intestinal inflammation with immunosuppressive medication such as corticosteroids, anti-TNF- α agents and other biologic inhibitors. The main features of CD and UC include increased intestinal permeability and increased mucosal levels of pro-inflammatory cytokines but the causality of this association remains uncertain (Michielan and D'Incà, 2015). On one hand, a subset of healthy first-degree relatives of CD patients exhibit increased intestinal permeability (Buhner et al., 2006; D'Incà et al., 2006; Fries et al., 2005) and at least in one case study increased intestinal permeability at the age of 13 preceded the onset of CD later in life (Irvine and Marshall, 2000). Increased intestinal permeability seems to also predict disease relapse in patients with inactive CD (Arnott et al., 2000; D'Incà et al., 1999). Furthermore, animal models of IBD, such as IL-10^{-/-}

and Samp/YitFc mice which spontaneously develop colitis and ileal inflammation, respectively, show increased intestinal permeability before the development of intestinal inflammation (Madsen et al., 1999; Olson et al., 2006). In IL-10^{-/-} mice, modulating intestinal permeability also affects the severity of the disease (Arrieta et al., 2015, 2009). These findings would suggest that defects in intestinal barrier function drive the pathogenesis of IBD.

On the other hand, anti-TNF- α antibody infliximab restores CD patients' intestinal permeability to the level of healthy controls (Noth et al., 2012; Suenaert et al., 2002) citing inflammation as the cause of increased intestinal permeability in these patients. Also, studies on different animal models have revealed that increased intestinal permeability alone does not always lead to overt intestinal inflammation. For example, JAM-A^{-/-} mice (Khounlotham et al., 2012), claudin-2 overexpressing mice (Ahmad et al., 2014), and mice expressing constitutively active MLCK (Su et al., 2009) all show increased intestinal permeability but do not develop colitis spontaneously. These models suggest that additional triggers besides increased intestinal permeability are needed for the development of IBD. Hence, the primary barrier defect in IBD likely results from abnormal mucosal immune response leading to subclinical inflammation which subsequently exacerbates the existing condition. This vicious cycle eventually leads to extensive intestinal inflammation and to the development of the disease. Most likely though, the interplay between inflammation and intestinal permeability involves also other factors (genetic, environmental, microbial) that impact the intestinal barrier and contribute to disease pathogenesis in IBD.

2.2.2 Intestinal microbes

Commensal microbes residing in the intestine regulate the intestinal barrier function via several mechanisms (Yu et al., 2012). Firstly, by inhabiting the intestinal lumen, commensal bacteria prevent the colonization of pathogenic bacteria. A good example of the protective impact of commensal microbiota is the possible expansion of pathogenic *Clostridium difficile* in the intestinal

REVIEW OF LITERATURE

lumen and subsequent diarrhea following the use of antibiotics (Deshpande et al., 2013). Secondly, intestinal microbiota metabolizes some of the undigested carbohydrates to short-chain fatty acids (SCFAs), such as butyrate. Butyrate is a major energy source for colonocytes and it has shown various beneficial effects for intestinal barrier function (for review, see Plöger et al., 2012). Interestingly, studies have reported decreased abundances of butyrate-producing bacteria in CD (Takahashi et al., 2016), UC (Machiels et al., 2014), IBS (Pozuelo et al., 2015), and type-1-diabetes (Vaarala, 2013), which are all conditions associated with increased intestinal permeability.

Thirdly, the direct interactions between commensal bacteria and the host are critical for maintaining the intestinal homeostasis. The intestinal epithelium contains different pattern-recognition receptors (PRRs) which recognize and respond to bacterial components, such as lipopolysaccharide (LPS), peptidoglycans, and flagellins (Martini et al., 2017). When these components bind to PRRs, they elicit the secretion of antimicrobial peptides, different cytokines, and other substances which modulate the immunological response and also shape the composition of intestinal microbiota (Fukata and Arditi, 2013). This immunomodulatory system has several implications for intestinal barrier function. For example, under germ-free conditions, colitis-prone IL-10^{-/-} mice do not develop permeability defects nor colitis indicating that abnormal immune response to intestinal microbes prompts the disease (Madsen et al., 1999). In humans, one of the major susceptibility loci for CD development is the gene for PRR called nucleotide-binding oligomerization domain-containing protein (NOD)-2 (Hugot et al., 2001) and a few studies have associated NOD2 gene variants with increased intestinal permeability in healthy relatives of CD patients (Buhner et al., 2006; D'Incà et al., 2006). Furthermore, NOD2^{-/-} mice exhibit increased intestinal permeability suggesting its role in maintaining intestinal barrier function (Al Nabhani et al., 2016). Another important PRR group in the context of intestinal permeability are toll-like receptors (TLRs). TLR signaling in the intestine forms a complex network with different receptors exerting pro-inflammatory and anti-inflammatory effects depending on the

receptor subtype and location (Fukata and Arditi, 2013). In mice, the deletion of TLR4 gene, a major receptor for bacterial LPS, prevents LPS-induced increase in intestinal permeability (Guo et al., 2015). On the other hand, TLR2 agonist ameliorates chemically-induced colitis in mice by restoring the intestinal barrier function (Cario et al., 2007).

Considering the interplay between PRRs and intestinal microbiota, abnormal PRR function likely contributes to intestinal dysbiosis. Intestinal dysbiosis is a frequently used, but loosely defined, term for reduced richness and diversity of intestinal microbiota and it is often considered as a sign of intestinal inflammation (Suchodolski, 2016). However, whether intestinal dysbiosis itself increases intestinal permeability remains uncertain. NOD2^{-/-} mice show increased intestinal permeability and intestinal dysbiosis but these two events seem to occur independently as NOD2^{-/-} associated-microbiota in wild-type control does not increase intestinal permeability (Al Nabhani et al., 2016). In contrast, studies in fruit flies have revealed that intestinal dysbiosis precedes intestinal barrier defect and this defect further exacerbates the dysbiosis (Clark et al., 2015). Similarly, a recent study in mice showed that microbial dysbiosis can increase intestinal permeability and the subsequent inflammation further contributes to the dysbiosis (Thevaranjan et al., 2017).

2.2.3 Dietary factors

The human diet contains several nutrients and molecules which can impact intestinal barrier function. In recent years, the relationship between intestinal permeability, obesity, and Western diet (a diet containing high amounts of fat and sugar) has been under extensive research. The current hypothesis states that increased intestinal permeability contributes to the pathophysiology of obesity-related diseases by allowing the translocation of LPS into circulation. This leads to a condition labeled metabolic endotoxemia which triggers the low-grade inflammation associated with obesity and obesity-related disorders. Multiple recent animal studies have reported increased intestinal permeability following a high-fat or high-fat/high-sugar diet (Cani et al., 2008; Hamilton et

REVIEW OF LITERATURE

al., 2015; Johnson et al., 2015; Martinez-Medina et al., 2014; Murakami et al., 2016; Serino et al., 2012; Stenman et al., 2012; Volynets et al., 2017) but whether this effect is caused by the diet or by diet-induced obesity remains uncertain. Studies on this have produced mixed findings with some reporting that genetically obese rodents exhibit increased intestinal permeability (Brun et al., 2007; Johnson et al., 2015) whereas others have reported normal permeability measures in these animals (Stenman et al., 2013b; Suzuki and Hara, 2010). Nonetheless, these findings are clinically relevant because they have linked increased intestinal permeability to the pathogenesis of obesity-related metabolic disorders, such as type-2-diabetes (T2D), nonalcoholic fatty liver disease (NAFLD), and nonalcoholic steatohepatitis (NASH). Indeed, obese people and patients with these diseases seem to be more likely to have increased intestinal permeability compared to healthy controls (Horton et al., 2014; Leber et al., 2012; Luther et al., 2015; Teixeira et al., 2012b).

Investigations into the mechanisms behind increased intestinal permeability in diet-induced obesity have revealed two major players: intestinal microbiota and bile acids. Regarding microbiota, Serino et al. (2012) showed that supplementing high-fat diet with gluco-oligosaccharide, a microbiota-modifying prebiotic, stunts both the increase in intestinal permeability and in blood LPS levels. Antibiotics have a similar effect (Cani et al., 2008) which further suggests that changes in microbiota contribute the diet-induced barrier defect. Another possible mechanism relates to bile acids. Bile acids facilitate the absorption of fatty acids from the intestine and their secretion is increased in response to high-fat diet. Stenman et al. (2012) reported that mice fed with high-fat diet exhibit altered fecal bile acid profile and this relates to increased intestinal permeability. Later, they showed that fecal concentrations of hydrophobic bile acid, deoxycholic acid (DCA), associated with high-fat feeding are able to disrupt the intestinal barrier (Stenman et al., 2013a). Considering that multiple studies have shown the barrier-disrupting effects of DCA (Chadwick et al., 1979; Fihn et al., 2000; Goerg et al., 1983; Hughes et al., 2008; Münch et al., 2007; Sun et al., 2004), it is likely that bile acids also contribute to

the observed increase in intestinal permeability during high-fat diet. However, it should be kept in mind that intestinal microbiota affects the composition of the bile acid pool and vice versa so it is highly probable that changes in one during high-fat diet also elicit changes in the other (Ridlon et al., 2014). Thus, more studies are needed on their synergistic effects in controlling intestinal barrier function during high-fat feeding.

Another disease associated with increased intestinal permeability and dietary habits is alcoholic liver disease (ALD). The etiology of ALD seems similar to that of NAFLD in that increased intestinal permeability permits the increased translocation of LPS into circulation and liver. Studies have shown that chronic and acute ethanol consumption increase intestinal permeability (Leclercq et al., 2017) and that changes in intestinal permeability may precede the onset of liver disease (Keshavarzian et al., 2009). Interestingly, alcoholics with chronic liver disease exhibit higher intestinal permeability than alcoholics with no liver disease suggesting that intestinal permeability plays a role in the development of ALD (Keshavarzian et al., 1999). The mechanisms by which ethanol disrupts the intestinal barrier involve oxidative stress and tight junctional modifications (Leclercq et al., 2017). In addition, the intestinal microbiota plays a role in ethanol-induced barrier defect as germ-free mice do not show increased intestinal permeability after acute alcohol consumption (Canesso et al., 2014). Recently, Leclercq et al. (2014) identified two subsets of alcoholics: those with increased intestinal permeability and those whose intestinal permeability was comparable to healthy controls. Intriguingly, intestinal dysbiosis was only present in the alcoholics with increased intestinal permeability and they also suffered greater levels of psychological symptoms during alcohol withdrawal (Leclercq et al., 2014). Also, when germ-free mice are inoculated with microbiota from an alcohol-dependent subject with severe liver disease, they exhibit greater intestinal permeability and liver damage after alcohol-feeding than comparable mice harboring microbiota from a similar subject without liver disease (Llopis et al., 2016). These results suggest that ethanol and gut microbiota act synergistically in the intestine to disrupt the barrier-function.

REVIEW OF LITERATURE

Of individual nutrients that affect intestinal permeability, gliadin, a component of grain protein gluten, has received a lot of attention due to its role in the pathogenesis of celiac disease. Gliadin increases intestinal permeability by inducing the secretion of zonulin (Lammers et al., 2008) (see, p. 39) and this response is greater in celiac patients (Drago et al., 2006). Increased intestinal permeability also seems to affect the development of food allergies (Järvinen et al., 2013; Ventura et al., 2006). Certain amino acids, such as glutamine, arginine, and tryptophan, have also shown barrier-enhancing properties when administered to different animal models (Andrade et al., 2015). In addition, vitamin D supplementation maintains intestinal permeability measures at remission level in CD patients (Raftery et al., 2015) possibly by suppressing MLCK-mediated tight junctional modifications (Du et al., 2015).

2.2.4 Psychological and physical stress

Acute and chronic psychological stress cause various physiological, metabolic, and behavioral alterations but also modulate the properties of the intestinal barrier. Different animal models of stress exhibit increased intestinal permeability and convergent evidence suggests that the primary mechanism behind this event involves corticotropin-releasing factor (CRF) signaling (Kelly et al., 2015; Rodiño-Janeiro et al., 2015). Hypothalamic CRF secretion is one of principal mediators of the systemic stress response and this central release of CRF also acts in the intestine by activating enteric CRF-receptors. Intestinal immune cells and enterochromaffin cells also produce CRF locally in the intestine (Rodiño-Janeiro et al., 2015). In the context of intestinal permeability, *in vivo* and *ex vivo* studies have shown that stress and exogenous CRF administration increase intestinal permeability via activation of intestinal mast cells and that pharmacological mast cell inhibitors block this effect (Lennon et al., 2013; Overman et al., 2012; Smith et al., 2010; Vanuytsel et al., 2014). Clinically, psychological stress has been associated with the development of IBD, IBS, and other gastrointestinal disorders (Brzozowski et al., 2016) which

highlights the role of gut-brain axis and the enteric nervous system in the maintenance of the intestinal barrier.

In addition to psychological stress, physical stressors, such as trauma and high-intensity exercise, have been shown to increase intestinal permeability. Although the mechanisms by which they compromise the intestinal barrier likely overlap (Clark and Mach, 2016; Hill et al., 2013), the main mechanism behind barrier defects during or after physical stress is gastrointestinal hypoperfusion and ischemia. Ischemia and the subsequent restoration of blood flow induce enterocyte damage, oxidative stress, and inflammation which compromise the integrity of the epithelium (van Wijck et al., 2012a). In trauma patients, intestinal cell damage is thought to facilitate bacterial translocation and sepsis which ultimately lead to multi-organ failure, a common cause of death in intensive care (Bischoff et al., 2014; Swank and Deitch, 1996). Regarding exercise, athletes frequently suffer from various gastrointestinal symptoms during training (Oliveira et al., 2014) and intestinal hypoperfusion is acknowledged as the leading cause behind these disorders (ter Steege et al., 2012; ter Steege and Kolkman, 2012). However, although several studies have reported increased intestinal permeability following high-intensity exercise (Davison et al., 2016; March et al., 2017; Marchbank et al., 2011; Oktedalen et al., 1992; Pals et al., 1997; van Nieuwenhoven et al., 2004; van Wijck et al., 2011; Zuhl et al., 2015), it remains unclear whether compromised barrier function contributes to gastrointestinal symptoms during exercise. Previously, van Nieuwenhoven et al. (2004) reported that symptomatic athletes exhibit higher intestinal permeability after exercise than asymptomatic athletes, but this comparison was based on historical asymptomatic controls who used a different mode of exercise. It is also unclear whether exercise-induced increase in intestinal permeability leads to elevated levels of LPS in circulation or if LPS mediates symptom development during exercise. Studies have reported increased blood concentrations of LPS following exercise (Ashton et al., 2003; Bosenberg et al., 1988; Gill et al., 2015; Jeukendrup et al., 2000; Lim et al., 2009; Moncada-Jiménez et al., 2009; Yeh et al., 2013) but aside from a single study

REVIEW OF LITERATURE

(Brock-Utne et al., 1988) it does not appear to directly correlate with the occurrence of gastrointestinal symptoms. These observations suggest that although increased intestinal permeability may play a role in exercise-induced gastrointestinal symptoms, more research is needed to elucidate the pathophysiological mechanisms that lead to symptoms in individual athletes.

2.2.5 Chemotherapy

Gastrointestinal toxicity is the most common adverse event associated with chemotherapeutic drugs (Andreyev et al., 2012). Chemotherapeutics are cytotoxic agents designed to kill rapidly dividing cells by inhibiting DNA replication with various mechanisms and thus interfering with cell division. For example, antimetabolites 5-FU and methotrexate disrupt the production of nucleotide thymidine needed for DNA synthesis. Platinum-based chemotherapeutics, such as cisplatin and oxaliplatin block DNA replication by forming adducts with the DNA strands. In contrast, irinotecan inhibits DNA topoisomerase-1, an enzyme needed during DNA replication. Regardless of the mechanism, chemotherapeutics disrupt the natural turnover of enterocytes and thus impact the intestinal mucosa. However, the pathophysiology of chemotherapy-induced gastrointestinal toxicity (CIGT) seems to be much more complicated and extend beyond simple cellular damage. Previously, Sonis (2004) proposed five overlapping phases for the development of CIGT. First, chemotherapeutics disrupt the cells' DNA resulting to cellular damage and to the generation of reactive oxygen species (*Initiation phase*). This initial damage activates transcription factors, such as nuclear factor- κ B, leading to the release of pro-inflammatory cytokines that further exacerbate the initial injury (*Primary damage response phase*). The release of pro-inflammatory cytokines creates a positive-feedback loop that further amplifies the damage (*Signal amplification phase*) eventually compromising the integrity of the intestinal barrier (*Ulceration phase*). Finally, when the chemotherapy is stopped, mucosal cells start to proliferate and the tissue heals (*Healing phase*) (Sonis, 2004). Recent evidence has expanded this model by introducing the effects of

intestinal microbiota to pathophysiology of CIGT (Alexander et al., 2017; van Vliet et al., 2010).

Indeed, multiple animal and clinical studies have reported increased intestinal permeability following the administration of different chemotherapeutics and chemotherapy-regimens (Beutheu et al., 2014; Choi et al., 2007; Daniele et al., 2001; Fazeny-Dörner et al., 2002; Generoso et al., 2015; Justino et al., 2014; Kato et al., 2017; Keefe et al., 1997; Lima-Júnior et al., 2014; Melichar et al., 2008, 2007; Russo et al., 2013; Vanhoecke et al., 2015; Wang et al., 2015; Wardill et al., 2016). The exact molecular mechanisms by which individual chemotherapeutics disrupt the intestinal barrier remain poorly defined. Overall, the molecular mechanisms of the pathophysiology of CIGT likely involve tight junctional modifications (Wardill and Bowen, 2013), inflammatory cascades (Cario, 2016; Lee et al., 2014), and gut microbiota modulations (Toucheffeu et al., 2014). The molecular mechanisms behind these events are best characterized with irinotecan while studies with other chemotherapeutics mainly describe increased inflammatory activity following chemotherapy. The pathophysiology of irinotecan-induced gastrointestinal toxicity has also been under interest due to irinotecan's unique metabolism by the intestinal microbiota (Alexander et al., 2017). Some bacterial species express an enzyme called β -glucuronidase that can metabolize irinotecan inactive, glucuronidated metabolite back to its active form and thus increase its toxicity. Recently, Pedroso et al. (2015) showed that germ-free mice mono-colonized with a bacterial strain that does not produce β -glucuronidase exhibit irinotecan-induced intestinal toxicity but not increased intestinal permeability. Also, considering that inhibiting this enzyme alleviates irinotecan-induced diarrhea (Wallace et al., 2010) and the efficacy of antibiotics (Bowen et al., 2007; Kurita et al., 2011) as well as pro- and prebiotics (Bowen et al., 2007; Lin et al., 2014) in reducing irinotecan's toxicity, the intestinal microbiota seems to be a vital at least for the pathophysiology of irinotecan-induced gastrointestinal toxicity.

REVIEW OF LITERATURE

To summarize, several factors impact the integrity of the intestinal barrier. Of special importance is the interplay between intestinal permeability, intestinal microbes, and inflammatory signals. It seems that factors capable of affecting this balance by either directly damaging the intestinal epithelium, modulating intestinal microbiota, or inducing inflammatory processes disrupt the intestinal homeostasis which starts a self-propagating cycle that results in more widespread changes and possibly disease.

2.3 How to measure intestinal permeability?

2.3.1 Direct permeability assays in vitro

Permeability assays in vitro are based on human colon tumorigenic cell lines which upon differentiation form enterocyte-like, polarized monolayers that resemble the intestinal epithelium (Cencic and Langerholc, 2010; Volpe, 2010). The most commonly used cell lines in these assays are Caco-2, HT-29, and T84 (Cencic and Langerholc, 2010). In permeability studies, these cells are cultivated on a semi-porous membrane separating the apical (luminal) and basolateral (submucosal) compartments. The macromolecular permeability of the monolayer can be assessed by introducing a probe molecule to the apical compartment and measuring the rate at which it translocates to the basolateral compartment. Frequently used probes in these assays are different sized fluorescein isothiocyanate (FITC)-labeled dextran and polyethylene glycol (PEG) polymers, fluorescein, Evans blue, and lucifer yellow due to their simple detection with a fluorescence counter. In addition to permeability probes, in vitro studies often use the TER value of the cell monolayer as a measure of its integrity.

2.3.2 The Ussing chamber for ex vivo permeability assays

Developed by a Danish biologist Hans Ussing in 1951 (Ussing and Zerahn, 1951), the Ussing chamber is still a widely used ex vivo approach for studying epithelial permeability (Clarke, 2009). A permeability assay in the Ussing chamber system requires a freshly harvested intestinal tissue segment that is mounted between two chamber halves separating the luminal and submucosal sides. To ensure the viability of tissue segment, each chamber half is filled with equal amounts of physiological buffer solution with continuous oxygenation and the whole system is water-jacketed to mimic mammalian body temperatures. Each chamber also contains two pairs of electrodes. The first set of electrodes measures the spontaneously developing voltage difference (V_t) across the tissue segment while the other electrode pair generates a current that nulls this

REVIEW OF LITERATURE

difference. This current is called the short-circuit current (I_{sc}) and it corresponds to the sum of all active ion movement across the epithelium. For an excellent review of the Ussing chamber set-up, see Clarke (2009).

Regarding permeability measurements in the Ussing chamber, the integrity of the paracellular pathway can be estimated from the TER values of the tissue segments. The TER values can be derived from the I_{sc} and V_t values according to the Ohm's law:

$$R = V/I$$

where the R is the resistance of the conductor in ohms (i.e. TER), the V is the voltage across the conductor in volts (i.e. the voltage difference between the two chambers), and the I is the current through the conductor in amperes (i.e. the I_{sc}). However, considering that the TER value mostly relates to paracellular ionic conductance regulated by the pore pathway (see Chapter 2.1.2., p. 16), changes in TER alone may not reflect changes in macromolecular permeability. In the Ussing chamber, macromolecular permeability can be assessed similarly as in *in vitro* studies by measuring the appearance of a permeability probe to the submucosal chamber from the apical side. The probes used in these studies are also similar to those used in *in vitro* studies. However, the major advantage of the *ex vivo* approach compared to the cell models is that the tissue segment retains the natural cellular diversity and complexity of the intestine. The limitations of the Ussing chamber are its invasiveness due to the requirement for intestinal tissue and the relatively short viability of intestinal tissue segments outside the body which limits measurement times. In addition, depending on the preparation of the tissue segment prior to mounting to the Ussing chamber, the intestinal specimen may include other anatomical structures besides to the epithelium which can form permeability barriers.

2.3.3 Direct permeability assays *in vivo*

In vivo measurements of intestinal permeability attempt to assess changes in intestinal barrier function on a systemic level. These assays are based on the oral administration of specific probe molecules and their subsequent analysis

from blood or urine. Most assays employ urine collection times ranging from 5-24 h with blood samples usually taken 1-2 hours after probe ingestion. Increased blood or urine concentrations of the probe indicate increased intestinal permeability suggesting impaired barrier function. This methodology poses several requirements for the probe molecules (for review, see Bjarnason et al., 1995). In general, they should be water-soluble, non-toxic, not actively absorbed from the intestine, not naturally present in the host, not metabolized, and easily detectable. A suitable probe for an individual study also depends on which segment of the gastrointestinal tract is under investigation (Arrieta et al., 2006).

The most frequently used probes for *in vivo* intestinal permeability measurements are small sugar molecules and disaccharides. Usually, the measurement is done by combining a small molecule (e.g. mannitol or rhamnose) with a disaccharide (e.g. lactulose) and intestinal permeability is expressed as the ratio between the two probes. The idea is that the smaller molecule acts as an internal standard eliminating individual differences in gastrointestinal motility and renal clearance. Currently, the lactulose/mannitol (L/M)-ratio is considered the golden standard for intestinal permeability measurements.

A unique feature in using sugar molecules as permeability probes is that they are susceptible to degradation in the intestine. For instance, colonic bacteria degrade both mannitol and lactulose meaning that their permeability reflects small intestinal permeability (Arrieta et al., 2006). On the other hand, sucrose is already metabolized in the duodenum making it a suitable probe for gastroduodenal permeability (Meddings et al., 1993). Whole gastrointestinal permeability can be assessed with sucralose or erythritol which are not degraded at any part of gastrointestinal tract. When sucralose is used together with lactulose, colonic permeability can be calculated by subtracting urinary lactulose excretion from urinary sucralose excretion (Bischoff et al., 2014). Furthermore, the individual differences between the degradation patterns of different sugar probes enables the use of several sugar probes in a single

REVIEW OF LITERATURE

measurement and assess gastroduodenal, small intestinal, and colonic permeability simultaneously (van Wijck et al., 2013). However, the use of sugar probes also possesses several limitations (for review, see Salles Teixeira et al., 2014). Small intestinal bacterial overgrowth or intestinal dysbiosis may impact measurements using sugars susceptible to bacterial degradation. Lactulose, mannitol, and sucralose are also present in multiple food products which requires careful monitoring of diet during the measurement period (Salles Teixeira et al., 2014). The analysis of sugars also requires complex detection methods, such as high-pressure liquid chromatography (HPLC) or liquid chromatography combined with mass spectrometry (LC/MS), which are not widely available.

Other suitable probes for in vivo permeability measurements include PEG polymers, ^{51}Cr -labeled ethylenediaminetetraacetic acid (^{51}Cr -EDTA), and iohexol (Salles Teixeira et al., 2014). All these probes pass through the intestinal tract non-metabolized and thus can be used for whole gut permeability assays. Similarly to sugar probes, PEG polymers require laborious detection methods (HPLC or LC/MS) and currently, they are not widely used in permeability studies. In contrast, the main advantage of using ^{51}Cr -EDTA is the radioactive label that makes its detection simple (Bjarnason et al., 1995). However, its radioactivity also makes it unsuitable for certain patients and may raise concerns about safety and proper handling of the probe. The use of radioisotopes can also be expensive and requires a permit which can further limit the use of ^{51}Cr -EDTA. Iohexol is a routinely used as a contrast medium in X-ray based imaging techniques but studies have shown that it is also suitable for intestinal permeability measurements (Andersen et al., 2001; Frias et al., 2014; Geroova et al., 2011; Halme et al., 2000, 1997, 1993). Compared to sugar probes, it is not affected by the intestinal microbiota and its detection can be done with a commercially available enzyme-linked immunosorbent assay (ELISA) kit. It is also non-radioactive, cheap, and due to its widespread use in clinical imaging it is readily available in clinical settings. However, studies using iohexol as a permeability marker are still rare and more studies are needed to

validate the method. Iohexol also contains iodine which prohibits its use in patients sensitive to iodine.

So far, intestinal permeability assays have not reached widespread use in clinical practice and they are mainly employed only for scientific investigations (Bischoff et al., 2014). This could be due heterogeneity in methodology between studies which has led to a lack of understanding of what constitutes as normal intestinal permeability in a certain situation. Also, direct permeability assays with urine collections times of 5-24 hours and laborious detection methods might be impractical to use in everyday clinical practice. Different probe molecules and their features are listed in Table 1.

REVIEW OF LITERATURE

Table 1. Frequently used probe molecules in direct permeability measurements (modified from Vancamelbeke and Vermeire, 2017).

Permeability probes	Use in permeability assays
Sugar molecules	
Mannitol Rhamnose	In vivo, used together with lactulose to measure small intestinal permeability, bacterial degradation, laborious detection
Sucrose	In vivo, degraded in the duodenum, reflects gastroduodenal permeability, laborious detection
Lactulose	In vivo, used to measure small intestinal permeability, bacterial degradation, laborious detection
Sucralose	In vivo, whole intestinal permeability measurements, laborious detection
Radioactive	
⁵¹Cr-EDTA	In vivo, whole intestinal permeability measurements, radioactivity a major disadvantage
Contrast medium	
Iohexol	In vivo, whole intestinal permeability measurements
Polymers	
Polyethylene glycols	In vivo, whole intestinal permeability measurements, laborious detection
Fluorescent	
Fluorescein Evans blue Lucifer yellow	In vitro and ex vivo permeability assays, easy detection
FITC-labeled dextrans	In vitro and ex vivo permeability assays, used also in vivo in rodents, easy detection

2.3.4 Indirect measures of intestinal barrier function

Studies have also employed several indirect markers to assess the integrity of intestinal epithelium. Although these markers might not directly represent increased macromolecular flux from the intestinal lumen into circulation, they could provide valuable information about gastrointestinal function and health.

Zonulin is the only known physiological modulator that specifically targets tight junctions and elevated blood levels of zonulin are considered as a sign of increased intestinal permeability. Indeed, increased concentrations of zonulin have been associated with the development of several diseases also associated with increased intestinal permeability (for review, see Sturgeon and Fasano, 2016). So far, studies have identified two major triggers for zonulin release: intestinal bacteria and gliadin. Gliadin-stimulated zonulin release is thought to contribute to pathophysiology of celiac disease because intestinal tissue samples from patients with celiac disease exhibit higher zonulin release and increased intestinal permeability following gliadin treatment (Drago et al., 2006). Another disease associated with elevated zonulin levels is T1D. In T1D patients and their relatives, increased zonulin concentrations correlated with *in vivo* permeability (Sapone et al., 2006) and in a diabetes-prone rat model zonulin blocker administration decreased intestinal permeability and diabetes incidence (Watts et al., 2005). In addition to T1D, elevated zonulin levels have been associated with obesity (Moreno-Navarrete et al., 2012; Zak-Gołąb et al., 2013) and obesity-related disorders, such as T2D (Zhang et al., 2014) and NAFLD (Pacifico et al., 2014). Recently, Morkkala et al. (2016) studied zonulin levels in overweight pregnant women and reported that increased zonulin levels associate with decreased richness of intestinal microbiota (Morkkala et al., 2016), circulating levels of LPS and inflammatory markers (Morkkala et al., 2017a), and increased risk of gestational diabetes (Morkkala et al., 2017b). However, more research is needed to elucidate the role of zonulin in the normal regulation of the intestinal barrier.

REVIEW OF LITERATURE

Increased levels of LPS are often interpreted to reflect increased intestinal permeability. However, other factors also contribute to circulating LPS concentrations (Teixeira et al., 2012a). For example, changes in the composition of intestinal microbiota may lead to elevated LPS production in the intestinal lumen and thus raise blood LPS levels without any changes in intestinal barrier function. Also, several physiological mechanisms clear LPS from circulation so individual differences in these mechanisms affect blood LPS concentrations. Studies have also shown that LPS can cross the intestinal epithelium transcellularly via chylomicron-mediated mechanisms. Thus, although circulation LPS levels may be a good indicator of the consequences of barrier defects, they should not be considered as a direct measure of intestinal permeability.

Fatty acid-binding proteins (FABPs) are proteins present in the apical membrane of mature enterocytes. The intestinal tract expresses three types of FABP: liver-type FABP (L-FABP), intestinal-type FABP (I-FABP), and ileal bile acid-binding protein (I-BABP) (Bischoff et al., 2014). Upon enterocyte damage, FABPs are released into circulation and increased blood concentrations of FABPs have been observed following abdominal tissue injury (Relja et al., 2010) and intestinal ischemia during exercise (ter Steege et al., 2012; van Wijck et al., 2011). Serum I-FABP levels also associate with the severity of villous atrophy in celiac disease (Adriaanse et al., 2013). Another marker of enterocyte function is citrulline. Citrulline is produced in enterocytes from glutamine and decreased levels of citrulline in blood reflect the loss of enterocyte mass. Citrulline has shown promise as a biomarker for chemotherapy-induced gastrointestinal mucositis (van Vliet et al., 2009).

Urinary excretion of tight junction protein claudin-3 has also been used to assess intestinal barrier function. Studies have reported elevated levels of claudin-3 after exercise (Yeh et al., 2013), in IBD patients (Thuijls et al., 2010a), and in necrotizing enterocolitis (Thuijls et al., 2010b).

REVIEW OF LITERATURE

Calprotectin is a frequently used fecal biomarker of intestinal inflammation. In clinical practice, fecal calprotectin is used to differentiate between IBDs and other functional gastrointestinal disorders, such as IBS (Sipponen and Kolho, 2015). Fecal calprotectin measurements are also used to monitor the disease activity in IBD (Sipponen and Kolho, 2015). However, as barrier defects may arise also independent of inflammation, fecal calprotectin should only be considered as a measure of intestinal inflammation.

To summarize, the basic concept of direct intestinal permeability assay is to measure the translocation of a probe molecule across the intestinal epithelium. This can be done in vitro using certain cell lines, ex vivo by harvesting tissue samples or biopsies or in vivo with probes that can be detected from blood or urine. Indirect biomarkers are also useful for assessing the health of the intestinal barrier but should not be taken as a measure of intestinal permeability.

3 AIMS OF THE STUDY

Increased intestinal permeability is a common feature in multiple pathologies. This thesis consists of two animal studies and a clinical study with each study focusing on different stressor impacting intestinal permeability. The first animal study (Studies I/II) investigated the association between intestinal permeability and chemotherapy-induced gastrointestinal toxicity. The second animal study was an ex vivo study examining the mechanism by which hydrophobic bile acid deoxycholic acid (DCA) affects intestinal permeability (Study IV). In the clinical study, we examined how intestinal permeability relates to gastrointestinal symptoms during exercise (Study III).

The specific aims were:

- I. To study how individual chemotherapeutics affect intestinal permeability and whether intestinal permeability to iohexol correlates with the severity of chemotherapy-induced gastrointestinal toxicity (CIGT). (Study I)
- II. To test the hypothesis that CIGT is associated with changes in fecal microbiota, and the metabolome of serum and urine. (Study II)
- III. To examine how chemotherapy-induced changes in the composition of fecal microbiota and the metabolome relate to CIGT and increased intestinal permeability. (Study II)
- IV. To analyze running-induced changes in intestinal permeability and other markers of gastrointestinal function and study their possible associations with gastrointestinal symptoms during running. (Study III)
- V. To elucidate the mechanisms by which DCA increases intestinal permeability. (Study IV)

4 MATERIALS AND METHODS

4.1 Experimental animals and study subjects

4.1.1 Experimental animals

For Study I/II, a total of 48 male Sprague–Dawley rats were obtained from Harlan (Udine, Italy) at the age of six weeks. The animals were acclimatized for 18 days before the commencement of experimental protocol. The animal experiment was approved by the National Animal Experiment Board (ESAVI/114/04.10.07/2015).

A total of 20 male c57Bl/6J OlaHsd mice (Harlan, UK) between the age of eight and ten weeks were included in Study IV. The animal experiment was approved by the National Animal Experiment Board (ESAVI/6806/04.10.03/2011).

All animals were housed in a specific pathogen-free rodent facility with a 12-h light/dark cycle in a room temperature of $22 \pm 2^{\circ}\text{C}$ and relative humidity of $55 \pm 15\%$. All experimental animals had free access to food (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Madison, WI, USA) and tap water.

4.1.2 Study subjects

For Study III, a total of 24 active runners were recruited via online recruitment posters. All suitable study subjects were asked to sign a written consent form to confirm their participation to the study. After drop-outs, the group of subjects completing the whole study protocol consisted of 9 males and 8 females between the age of 24 and 44. Exclusion criteria included gastrointestinal illness, asthma, heart or cardiovascular diseases, pregnancy, breast-feeding, and iodine allergy. The study was conducted in adherence to the ethical regulations outlined in the Declaration of Helsinki and the study was approved by the Hospital District of Helsinki and Uusimaa (HUS) Coordinating ethics committee (13/13/03/00/2015).

MATERIALS AND METHODS

4.2 Study designs

4.2.1 Study I/II

Following the 18-day acclimatization period, the rats were randomized into four experimental groups: Control, 5-fluorouracil (5-FU), Oxaliplatin, and Irinotecan. Baseline samples of serum, urine, and feces were collected and a baseline measurement of in vivo intestinal permeability to iohexol was performed before any drug injections.

After baseline sample collection, the animals could recuperate for 13 days after which they received a single intraperitoneal injection of either 0.9% saline solution, 150 mg/kg 5-fluorouracil (5-FU; Accord Healthcare, Middlesex, UK), 15 mg/kg oxaliplatin (Hospira UK, Warwickshire, UK), or 200 mg/kg irinotecan (Hospira UK, Warwickshire, UK) (Table 2). Prior to the irinotecan administration, the animals were injected with 0.01 mg/kg atropine (Leiras, Espoo, Finland) subcutaneously to reduce the irinotecan-induced cholinergic reaction.

Table 2. Study groups and drug injections in Study I/II

Groups	n	Drug injection (i.p.)
Control	12	0.9% saline
5-FU	12	150 mg/kg 5-fluorouracil
Oxaliplatin	12	15 mg/kg oxaliplatin
Irinotecan	12	200 mg/kg irinotecan (+ 0.01 mg/kg s.c. atropine)

All animals were euthanized after a 72-h observation period during which they were weighted and scored for signs of diarrhea daily. Intestinal permeability measurement was started 48 h after drug injections. At euthanasia, serum, urine, fecal, and intestinal tissue samples were collected for later analyses. The study design is depicted in Figure 4.

4.2.2 Study III

In Study III, the study subjects were allocated into two groups: those who reported to experience gastrointestinal symptoms at least 50% of their runs (symptomatic, $n = 8$) and those who said to experience gastrointestinal symptoms in less than 10% of their runs (asymptomatic, $n = 9$) (Table 3). Intestinal permeability was measured at baseline and after a running test where the subjects were asked to run for 90 minutes at a speed equivalent to 80% of their best 10 km race time. Serum samples were collected by a licensed physician at baseline and immediately after the running test. The subjects were also asked to provide a fecal sample at baseline and after the running test. The study design is depicted in Figure 4.

Table 3. Study groups and criteria for group allocations in Study III

Groups	n	Criteria for group allocations
Asymptomatic	9	Experiences gastrointestinal symptoms < 10% of runs
Symptomatic	8	Experiences gastrointestinal symptoms \geq 50% of runs

4.2.3 Study IV

In Study IV, murine jejunal tissues were harvested and mounted in Ussing chambers for the measurement of intestinal permeability and electrophysiological parameters. Both intact jejunal segments and mucosal segments stripped of the seromuscular layer were treated with 3 mM DCA (Sodium deoxycholate; Sigma-Aldrich, St. Louis, MO, USA) on the luminal side for 20 min after which tissue permeability to fluorescein was measured for 105 min. In a set of experiments, prior to DCA-treatment, the intestinal segments were treated with 1 μ M tetrodotoxin (TTX; Tetrodotoxin citrate; Ascent Scientific, Bristol, UK) for 20 min to eliminate neural activity (Table 3). The study design is illustrated in Figure 4.

MATERIALS AND METHODS

Table 4. Study groups and in vitro treatment in Ussing chamber in Study IV

Groups	n	In vitro treatment in Ussing chamber
Mucosal	12	-
Mucosal + DCA	12	3 mM DCA for 20 min
Intact	22	-
Intact + DCA	22	3 mM DCA for 20 min
Intact + TTX	10	1 μ M TTX for 20 min
Intact + TTX +DCA	10	1 μ M TTX for 20 min + 3 mM DCA for 20 min

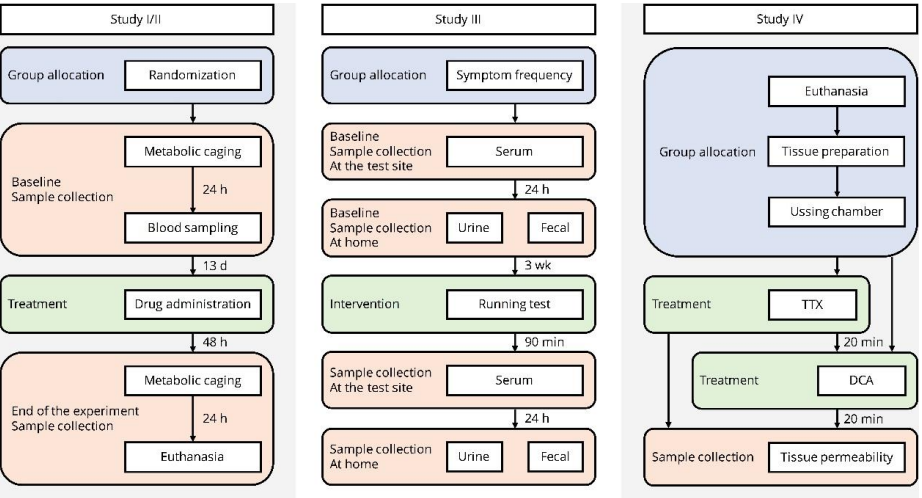


Figure 4. Flow charts illustrating the study designs of Studies I/II, III, and IV.

4.3 Sample collection and analyses

4.3.1 Measurement of in vivo intestinal permeability

In vivo intestinal permeability to iohexol was measured in Studies I/II and III.

In Study I/II, the rats received 1 ml of 647 mg/ml iohexol solution (Omnipaque 300™, GE Healthcare, Oslo, Norway) by oral gavage after which they were immediately placed in individual metabolic cages. After 24 h, the total amount of excreted urine was measured and stored in -18°C for the analysis of iohexol.

In Study III, the study subjects ingested 50 ml of 755 mg iohexol/ml solution (Omnipaque 350™, GE Healthcare, Oslo, Norway) dissolved in tap water at the baseline measurements and just before the running test. They were given specimen containers in which they were instructed to collect all excreted urine for 24 h at home. Upon returning the specimen containers, the total amount of excreted urine was measured, stirred, and a 5-ml sample was taken and stored in -18°C for iohexol analysis.

The concentration of urinary iohexol was analyzed using a commercial ELISA kit according to the manufacturer's instructions (BioPAL Inc., Worcester, MA, USA). Intestinal permeability to iohexol was determined by calculating the percentage of excreted iohexol using the following equation:

$$\text{Iohexol (\%)} = \frac{\text{amount of iohexol excreted in urine during 24 h (mg)}}{\text{amount of administered iohexol (mg)}} * 100$$

4.3.2 Tissue preparation for the Ussing chamber

Following euthanasia, fresh segments of murine jejunum were harvested by removing the entire small intestine and dissecting the four middle-most segments. The segments were pinned onto a Sylgard dish with ice cold Ringer solution (120 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 1.8 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, 1.25 mM CaCl₂, 1 mM MgSO₄, and 10 mM glucose), removed of mesenteric fat, and opened along the mesenteric border. After flushing the segments free of any intestinal content, the jejunal tissues were either mounted into the Ussing chamber system (intact samples) or stripped of their

MATERIALS AND METHODS

seromuscular layer (mucosal samples). The seromuscular stripping was done by pinning the intestinal segment tightly onto the Sylgard plate with the seromuscular layer facing upwards. The muscle layer was then carefully removed with fine forceps under a stereomicroscope. Immediately after seromuscular stripping, the mucosal tissue segments were pinned onto 0.3 cm² sliders and mounted into an Ussing chamber system with a voltage-clamp apparatus (EasyMount, Physiological Instruments, San Diego, CA, USA).

4.3.3 Measurement of in vitro intestinal permeability in the Ussing chamber

After the prepared tissue segments were mounted onto the Ussing chambers, each chamber half was filled with 5 ml Ringer solution to fully immerse the tissues in physiological solution. The whole system was water-jacketed to +37°C and a carbogen (95% O₂ – 5% CO₂, AGA, Riihimäki, Finland) flow was applied to each chamber. The system could equilibrate for 10 min before the start of any protocols.

Sodium fluorescein (Sigma-Aldrich) was added to the luminal chamber to a final concentration of 2 µg/ml. Tissue permeability was measured by taking a sample from the serosal chamber at 15, 45, 75, and 105 min and detecting sample fluorescence with a Wallac Victor² 1420 Multilabel counter (Perkin-Elmer, Waltham, MA, USA) using an excitation/emission wavelengths of 485/535 nm. The concentration of fluorescein was calculated from a standard curve and total tissue permeability to fluorescein was determined by establishing an apparent permeability coefficient (P_{app}) for each tissue segment using the following equation:

$$P_{app} = \left(\frac{dc}{dt} \right) * \left(\frac{V}{A * C} \right)$$

,where dc/dt is the change in concentration per unit time in the serosal chamber, V is the volume of the serosal chamber (5 ml), A is the surface area of the tissue (0.3 cm²), and C is the initial concentration of fluorescein in the luminal chamber (2 µg/ml). The P_{app} value of an individual tissue segment was

obtained by averaging the measured Papp values in each time point (15, 45, 75, and 105 min).

4.3.4 Measurement of electrophysiological parameters in the Ussing chamber

All Ussing chamber experiments were carried out under voltage-clamp conditions. Short-circuit current (Isc) and transepithelial resistance (TER) were monitored and recorded throughout each experiment with Acquire and Analyze 2.3 software (Physiological Instruments). The Isc was generated through Ag/AgCl electrodes connected to the chamber halves via 4% agar-3 M KCl salt bridges. Every 20 s the system was pulsed with a 5-mV step change in voltage-clamp mode and the resulting change in Isc was used to obtain the TER value of the tissue. The Isc and TER values for each time point were acquired by averaging the Isc and TER values between two adjacent time points.

4.3.5 Tissue collection for histological analysis

Intestinal tissue was collected for histological analyses in Studies I/II and IV.

In Study I/II, the entire intestine was removed and 1 cm segments from the middle of the jejunum as well as the colon were dissected. The tissue segments were cut open and flushed free of intestinal content with cold phosphate-buffered saline (PBS) solution.

In Study IV, the jejunal tissues were prepared as described in chapter 4.3.2. Tissue preparation for the Ussing chamber.

For histological analysis, the tissues were fixed in 10% neutral buffered formaldehyde (Sigma-Aldrich) for 24–48 h, embedded in paraffin, cut on microscopic slides, and stained with hematoxylin–eosin (HE).

4.3.6 Histological analysis

Detailed histological analysis of jejunum and colon was performed in Study I/II. The analysis was made in a partly blinded manner by a trained pathologist from the Faculty of Veterinary Medicine, University of Helsinki. Histological images

MATERIALS AND METHODS

were acquired with Axio Imager. A2 microscope (20x objective; Carl Zeiss, Goettingen, Germany).

The jejunal samples were analyzed for six change categories: villous stunting, villous epithelial injury, crypt hyperplasia, crypt epithelial injury, Paneth cell injury, and leukocyte infiltration in lamina propria. Comparable change categories were employed for the colon: surface epithelial injury, crypt hyperplasia, crypt dilatation and distortion, crypt epithelial injury, crypt loss (atrophy), and leukocyte infiltration in lamina propria. Each change category was scored on a five-tier scale: no changes (0), minimal (1), mild (2), moderate (3) and marked (4). Additionally, we created an overall score for acute jejunal and colonic injury. For acute jejunal injury, the grades of villous epithelial injury, villous stunting, crypt epithelial injury, and Paneth cell injury were combined and averaged. Similarly, the grades of surface epithelial injury, crypt epithelial injury, and crypt loss were combined and averaged to obtain a score for acute colonic injury.

4.3.7 Immunofluorescence analysis for enteric nerves

Immunofluorescence assay for the presence of enteric nerves in jejunal tissues was performed in Study IV.

First, the jejunal tissue samples were deparaffinized and boiled in 10 mM citrate buffer for 15 min for antigen retrieval. After washing the samples with PBS twice for 5 min, the sections were blocked with 5% normal goat serum (NGS) in PBS-T (0.3% Triton X-100) for 1 h at room temperature. Next, mouse monoclonal 13AA8 antineurofilament primary antibody was added in a 1:10 dilution (Virtanen et al., 1985; Ylikoski et al., 1990) in PBS-T with 1% NGS for overnight at +4°C. The tissue segments were then washed with PBS-T (3 * 10 min) before the addition of the secondary antibody goat antimouse-Alexa 488 (1:1000 dilution; Invitrogen, Carlsbad, CA, USA) in PBS-T with 1% NGS for 1 h at room temperature. Once again, the sections were washed with PBS-T (3 * 10 min) after which they were covered with 50% glycerol, and imaged with a fluorescence microscope (Axio Imager M2; Carl Zeiss, Oberkochen, Germany).

4.3.8 Global metabolome analysis of serum and urine

Global metabolic changes were analyzed from serum and urine with 1-mm proton nuclear magnetic resonance (^1H -NMR) technique in Study I/II.

The serum samples were prepared for ^1H -NMR analysis by mixing 20 μl of serum with 2.5 μl 2.5 mM sodium-3'-trimethylsilylpropionate-2,2,3,3- d_4 (TSP) in deuterium oxide (D_2O). For urine samples, 2 μl of a phosphate buffer solution (0.06 M Na_2HPO_4 / 0.04 M NaH_2PO_4 , pH 7) and 2.5 mM TSP were added to overcome the pH variation problem. The reason for the addition of TSP into the mixture is that it acts as standard compound in chemical shift referencing because TSP signal is always in the same position and does not overlap with other signals. Thus, other signals can be assigned relative to TSP signal.

^1H -NMR spectral acquisition was done with a standard one-dimensional pulse sequence with water suppression (Bruker Avance 600 spectrometer operating at 600.13 MHz with a 1-mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ TXI probe) at a nominal temperature of 310K. The acquired spectra were processed using MestReNova 8.1 (Mestrelab Research S.L., Spain). The resonances between 0.50-9.5 ppm were binned into 0.01-ppm buckets, mean-centered for multivariate analysis, and normalized to total aliphatic spectral area (0.50-4.30 ppm) to eliminate differences in total metabolite concentration. Peaks were assigned to their corresponding metabolites using data from literature and a commercial resonance database Chenomx (Chenomx NMR 7.6). Peak areas were integrated to calculate the relative concentrations. Overall, a total of 66 and 111 spectral regions for serum and urine, respectively, were transferred to MATLAB (MathWorks) for data analysis.

4.3.9 Fecal microbiota analysis

The composition of fecal microbiota was analyzed using 16S rRNA gene sequencing in Study I/II. Fecal samples were collected from the metabolic cage at baseline and at the end of experiment and stored in -80°C before analysis.

MATERIALS AND METHODS

The microbial DNA from rat fecal samples was purified using a commercial kit (QIAamp DNA Minikit, Qiagen, Doncaster, UK). The total DNA concentration of each sample was determined with Quant-iT™ PicoGreen® Assay (Invitrogen, Eugene, OR, USA) and the samples were diluted to a concentration of 1 ng/μl prior to 16S rRNA gene sequencing on the Illumina HiSeq2500 platform.

After a two-step PCR amplification protocol, the Illumina Nextera Kit was used to construct the DNA libraries. Paired-end sequencing of 200-nt-reads was performed. In total, the sequencing produced 9 698 319 reads which were processed using the R package *mare* (Korpela, 2016; R Development Core Team, 2008). The reads were filtered for quality and chimeras (UCHIME in the *de novo* mode) (Edgar et al., 2011) and subsequently annotated taxonomically using UPARSE (confidence cut-off at 0.5) (Edgar, 2013) with RDP as the reference database.

4.3.10 Biochemical analyses

Serum concentration of zonulin was measured using commercial ELISA kits (Rat: BlueGene, Shanghai, China; Human: Immunodiagnostik, Bensheim, Germany) in Studies I/II and III.

Serum concentration of intestinal fatty acid-binding protein (I-FABP) was analyzed using a commercial ELISA kit (Hycult Biotech, Uden, the Netherlands) in Study III.

Serum bacterial lipopolysaccharide (LPS) activity was quantified using a commercial Limulus Amebocyte Lysate (LAL) Chromogenic Endpoint Assay (Hycult biotech) in Study III.

In Study III, the study subjects were given written instructions to collect a fecal sample at home in a provided specimen container. Fecal concentration of calprotectin was measured with Calpro ELISA test (Calpro AS, Oslo Norway).

4.3.11 Assessment of clinical signs of gastrointestinal distress

In Study I/II, the animals were checked for diarrhea daily. The severity of diarrhea was scored on four-tier scale: no diarrhea (0), mild diarrhea (1), moderate diarrhea (2), and severe diarrhea (3). Grading was based on the extent of fecal staining and on the viscosity of the fecal matter.

In Study III, the assessment of gastrointestinal symptoms was performed in the form of questionnaires. At the beginning of the study, the study subjects filled out a background questionnaire probing the frequency of gastrointestinal symptoms during running. The subjects were also asked to rate their typical stool consistency after running. The results of this questionnaire were used to allocate the subjects to the two study groups. After the running test, the participants filled out another questionnaire asking if they had experienced gastrointestinal symptoms during the running test and if they experienced symptoms later that day. They also graded the amount of stomach pain they experienced during the running test on a visual analog scale (VAS) from 0 to 10 and rated the consistency of the first stool after the running test.

4.3.12 Data analyses

Statistical analyses were performed with PASW Statistics software versions 18.0.2 and 22.0 (IBM, Armonk, NY, USA) unless stated otherwise. All data were deemed significant when $P < 0.05$.

In Studies I/II and IV, the differences in measured variables (excluding fecal microbiota and metabolic analyses) between groups were first analyzed with non-parametric Kruskal-Wallis test and if global $P < 0.05$, the differences between individual groups were calculated using the Mann-Whitney U-test. In Study III with only two experimental groups, the differences between groups were analyzed using independent samples t-test. Paired t-test was used to analyze the changes within groups from baseline to after the running test.

The statistical analysis of the fecal microbiota data was performed using R package *mare*. The *mare* package was used to create the taxonomic tables, count

MATERIALS AND METHODS

the relative abundances of each taxon, and calculate the inverse Simpson diversity index. Treatment-induced changes in the relative abundances of bacterial taxa from baseline were identified using the change test script with false discovery rate correction (FDR) in the mare package. The Kruskal-Wallis test with Dunn-Bonferroni post hoc test was used to analyze the differences in the relative abundances of bacterial taxa between the groups at baseline and at the end of the experiment. Group differences in inverse Simpson diversity index were analyzed with one-way ANOVA followed by Bonferroni post hoc test.

Multivariate paired analysis with Bonferroni correction was used to calculate the differences in relative metabolite concentrations between the groups. To visualize the metabolic alterations between groups, principle component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed using in-house MATLAB scripts and PLS Toolbox (Eigenvector Research, Manson, WA, USA). The variable importance in the projection (VIP) scores were obtained from the PLS-DA model and variables with a VIP score ≥ 1 were considered relevant for group discrimination.

MATERIALS AND METHODS

Table 5. Summary of collected samples and measured variables in each study

	Study I/II	Study III	Study IV
Species (n)	Rat (48)	Human (17)	Mouse (11)
Study type	In vivo	In vivo	In vitro
Samples	Serum Urine Feces Intestinal tissue	Serum Urine Feces	Jejunal tissue
Measured variables	<u>Clinical signs:</u> Body weight change Severity of diarrhea <u>Serum:</u> Zonulin Global metabolome <u>Urine:</u> Intestinal permeability to iohexol Global metabolome <u>Feces:</u> Fecal microbiota composition <u>Intestinal tissues:</u> Histological analyses of jejunum and colon	<u>Clinical signs:</u> Occurrence of gastrointestinal symptoms Severity of stomach pain Stool consistency <u>Serum:</u> Intestinal fatty acid-binding protein (I-FABP) Bacterial lipopolysaccharide activity (LPS) Zonulin <u>Urine:</u> Intestinal permeability to iohexol <u>Feces:</u> Calprotectin	<u>Electrophysiological parameters:</u> Short-circuit current (Isc) Transepithelial resistance (TER) <u>Tissue permeability:</u> Permeability to fluorescein

RESULTS

5 RESULTS

5.1 Clinical signs of gastrointestinal distress

5.1.1 Chemotherapeutics induce body weight loss and diarrhea

All the administered chemotherapeutics induced significant ($P < 0.001$) body weight loss in the experimental animals during the 72-h experimental protocol compared to the Control group (Figure 5). Irinotecan administration reduced the animals' body weight by $16.0 \pm 3.5\%$ which was significantly ($P < 0.001$) more compared to either 5-FU ($6.6 \pm 2.3\%$) or oxaliplatin ($11.6 \pm 3.4\%$) administration. The Control group maintained their body weight during the experiment.

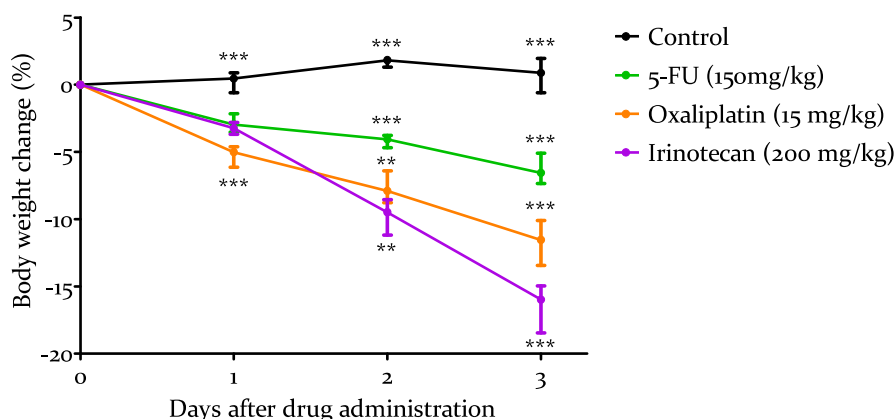


Figure 5. A single intraperitoneal injection of 5-fluorouracil (5-FU), oxaliplatin, or irinotecan on Day 0 significantly decreased the animals' body weight (%) already after 24 hours (Day 1). At the end of the experiment on Day 3, the animals in the Irinotecan group had lost significantly more of their body weight than animals that received either 5-FU or oxaliplatin. Oxaliplatin also induced significantly more severe body weight loss than 5-FU. Line graph shows medians with interquartile ranges. *** $P < 0.001$ compared to all other groups, ** $P < 0.01$ between groups. $n = 12$ in all groups, except in Oxaliplatin where $n = 10$. Modified from a figure originally appearing in *Cancer Chemotherapy and Pharmacology* (78:863-874, 2016) and reprinted under the Creative Commons license.

Most of the animals that received chemotherapeutic agents also exhibited diarrhea over the course of the experiment (Figure 6). In the Irinotecan group, 67% of the animals developed severe diarrhea, 25% moderate diarrhea, and 8% mild diarrhea. Animals that received oxaliplatin exhibited mainly moderate diarrhea (80%) with one case of both mild and severe diarrhea. In the 5-FU group, 50% of the animals had mild diarrhea. The Control group did not show any signs of diarrhea.

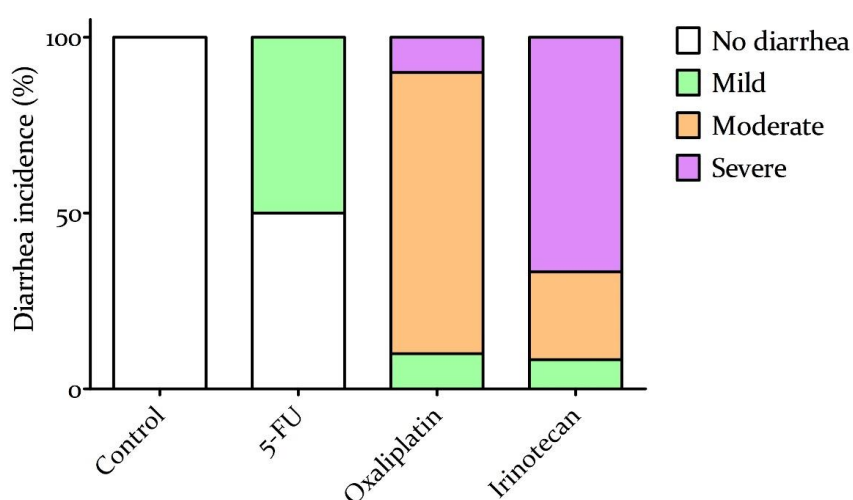


Figure 6. Diarrhea incidence (%) in the experimental groups over the course of the experiment (72 h). Administration of 5-FU induced mild diarrhea in half of the animals whereas most of the animals in the Oxaliplatin and Irinotecan groups exhibited moderate or severe diarrhea. $n = 12$ in all groups, except in Oxaliplatin where $n = 10$. Modified from a figure originally appearing in *Cancer Chemotherapy and Pharmacology* (78:863-874, 2016) and reprinted under the Creative Commons license.

RESULTS

5.1.2 Gastrointestinal symptoms during and after a 90-min running test

Overall, 3/9 runners in the asymptomatic group and 5/8 runners in the symptomatic group reported some degree of stomach pain during the running test. When scored on the VAS, the symptomatic runners had an average stomach pain of 2.0 ± 2.1 which was higher than asymptomatic runners (0.30 ± 0.56) but the difference did not reach statistical significance ($P < 0.061$) (Figure 7).

In the symptom questionnaire, all symptomatic runners and 5/9 asymptomatic runners reported to have experienced at least one gastrointestinal symptoms during or following the running test. Results of the symptom questionnaire are summarized in Table 6.

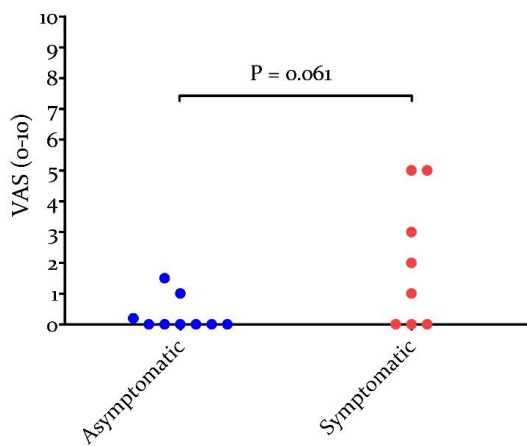


Figure 7. A total of five symptomatic runners and three asymptomatic runners reported at least some degree of stomach pain (VAS, 0-10) during the 90-min running test at a challenging pace.

Table 6. The occurrence of gastrointestinal symptoms during and following the running test

Symptom	Asymptomatic	Symptomatic
Nausea	0/9	3/8
Burping	1/9	3/8
Heartburn	0/9	1/8
Throwing up	0/9	0/8
Bloating	0/9	2/8
Liquid in stomach	0/9	1/8
Flatulence	4/9	4/8
Diarrhea	1/9	3/8
Constipation	1/9	2/8
Subjects reporting at least one symptom	5/9	8/8

5.2 Histological analyses of intestinal tissues

5.2.1 Chemotherapeutics disrupt intestinal morphology

Administration of the chemotherapeutic agents to the experimental animals resulted in significant tissue damage in both the jejunum and the colon (Table 7). Based on the acute injury score (Figure 8), oxaliplatin produced significantly ($P < 0.001$) more histopathological changes in the jejunum than 5-FU and irinotecan whereas irinotecan was significantly more toxic in the colon than oxaliplatin ($P < 0.05$) and 5-FU ($P < 0.001$). No histological changes were observed in the Control group.

RESULTS

Table 7. Histological grades of chemotherapy-induced tissue damage

Change category	5-FU	Oxaliplatin	Irinotecan
Jejunum			
Villous stunting	2.8 ± 1.1 ^a	3.6 ± 0.52 ^{a,b}	2.7 ± 0.65 ^b
Villous epithelial injury	2.3 ± 1.3 ^a	3.5 ± 0.70 ^{a,b}	2.3 ± 0.45 ^b
Crypt hyperplasia	0.75 ± 1.1 ^b	1.5 ± 1.1 ^a	2.5 ± 0.52 ^{a,b}
Crypt injury	2.9 ± 0.79 ^a	4.0 ± 0.0 ^{a,c}	2.7 ± 0.49 ^c
Paneth cell injury	2.3 ± 0.49 ^{a,c}	2.8 ± 0.42 ^{a,c}	0 ^c
Lamina propria leukocytes	2.9 ± 0.51 ^{b,c}	4.0 ± 0.0 ^c	1.6 ± 1.2 ^{b,c}
Colon			
Surface epithelial injury	1.3 ± 0.62 ^{a,c}	1.9 ± 0.87 ^a	2.3 ± 0.49 ^c
Crypt hyperplasia	1.7 ± 0.78	2.0 ± 0.0 ^a	1.0 ± 1.0 ^a
Crypt dilatation and distortion	2.3 ± 0.65	1.8 ± 0.63	2.2 ± 0.39
Crypt injury	1.8 ± 0.45 ^b	2.2 ± 0.63	2.7 ± 0.65 ^b
Crypt atrophy	0 ^b	0 ^b	0.83 ± 1.0 ^b
Lamina propria leukocytes	0.33 ± 0.78 ^c	0.80 ± 1.0 ^a	1.9 ± 0.67 ^{a,c}

Values are expressed as means ± standard deviations. 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. ^a*P* < 0.05 between groups, ^b*P* < 0.01 between groups, ^c*P* < 0.001 between groups.

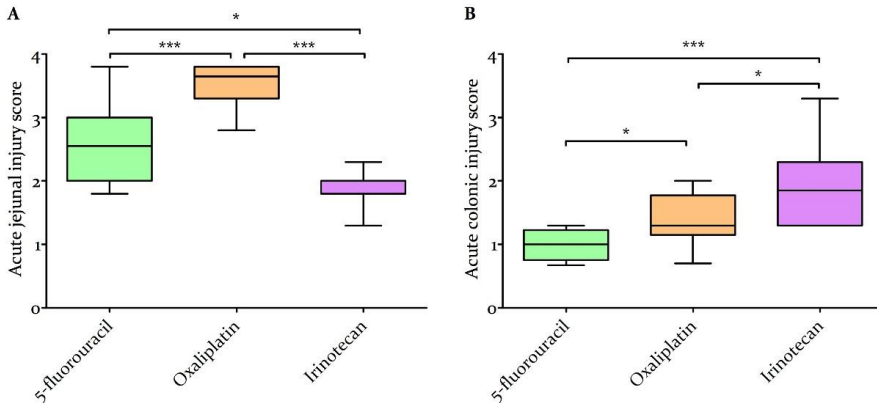


Figure 8. After histological examination of the intestinal tissues, acute jejunal (A) and colonic (B) injury scores were calculated for each treatment group. Oxaliplatin caused significantly more acute injury in the jejunum than 5-FU and irinotecan. In the colon, however, irinotecan administration produced the most severe damage. Box plots show median with upper and lower quartiles. Whiskers represent minimum and maximum. * $P < 0.05$, *** $P < 0.001$. $n = 12$ in all groups, except in Oxaliplatin where $n = 10$. Modified from a figure originally appearing in *Cancer Chemotherapy and Pharmacology* (78:863-874, 2016) and reprinted under the Creative Commons license.

5.3 Measurements of intestinal permeability

5.3.1 Chemotherapeutics increase in vivo intestinal permeability to iohexol

Intestinal permeability to iohexol was significantly ($P < 0.001$) increased in all chemotherapy-treated groups compared to the Control group (Figure 9). In addition, the Irinotecan group ($8.1 \pm 8.9\%$) exhibited significantly ($P < 0.001$) higher iohexol permeability than animals that received 5-FU ($1.6 \pm 1.5\%$) or oxaliplatin ($2.6 \pm 1.5\%$). At baseline, there were no differences in iohexol permeability between the groups (data not shown).

RESULTS

Iohexol permeability showed an inverse correlation with chemotherapy-induced body weight loss (Spearman's $\rho = -0.873$, $P < 0.001$) as well as a positive correlation with both diarrhea scores (Spearman's $\rho = 0.815$, $P < 0.001$) and acute colonic injury scores (Spearman's $\rho = 0.807$, $P < 0.001$) (Figure 10). There was also a positive correlation between acute jejunal injury score and iohexol permeability (Spearman's $\rho = 0.525$, $P < 0.001$) but this correlation exhibited a very low coefficient of determination ($r^2 = 0.05$, $P < 0.13$) indicating low dependency between the two variables (Figure 10C).

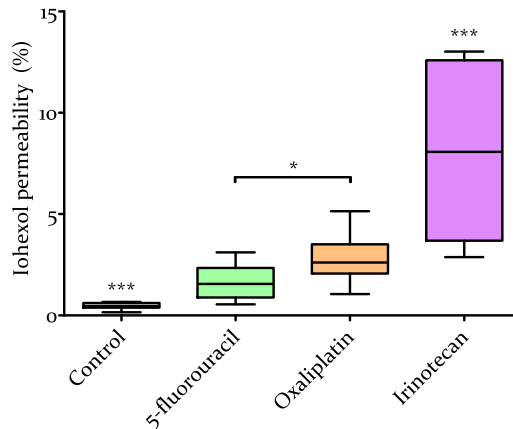


Figure 9. Intestinal permeability to iohexol (% of administered iohexol) at the end of experiment (72 h). Animals that received chemotherapeutics exhibited significantly higher iohexol permeability compared to the control animals. Of the studied chemotherapeutics, irinotecan induced the highest increase in iohexol permeability. Box plots show median with upper and lower quartiles. Whiskers represent minimum and maximum. * $P < 0.05$ between groups, *** $P < 0.001$ compared to all other groups. $n = 12$ in Control, $n = 11$ in 5-FU, $n = 10$ in Oxaliplatin, and $n = 9$ in Irinotecan. Modified from a figure originally appearing in *Cancer Chemotherapy and Pharmacology* (78:863-874, 2016) and reprinted under the Creative Commons license.

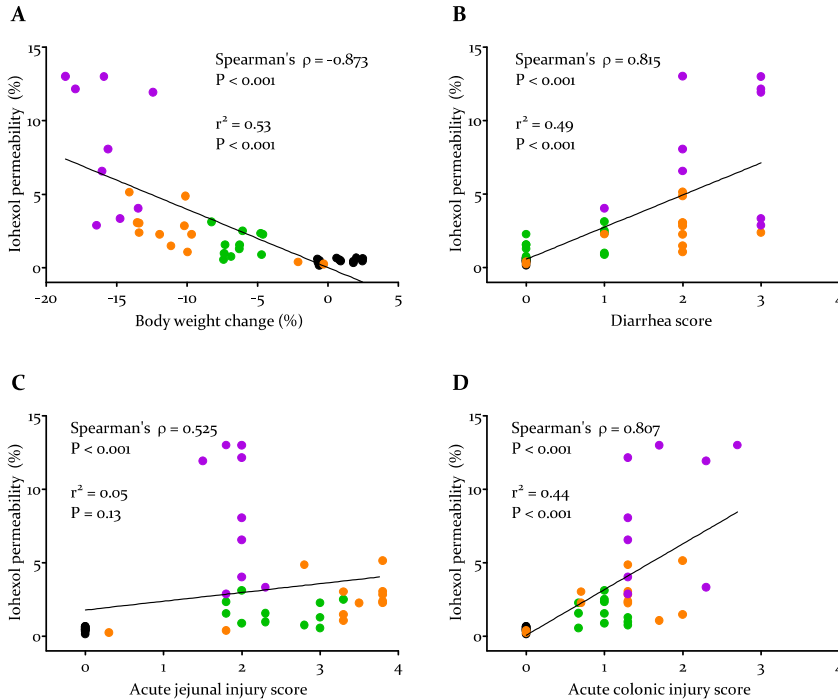


Figure 10. Spearman's correlations between iohexol permeability (%) and body weight change (A), diarrhea scores (B), acute jejunal injury scores (C), and acute colonic injury scores (D). Black dots represent samples from the Control group, green dots from the 5-FU group, orange dots from the Oxaliplatin group, and purple dots from the Irinotecan group. Modified from a figure originally appearing in *Cancer Chemotherapy and Pharmacology* (78:863-874, 2016) and reprinted under the Creative Commons license.

5.3.2 Running increases in vivo intestinal permeability to iohexol

Running for 90 minutes at a challenging pace significantly ($P < 0.001$) increased intestinal permeability to iohexol (Figure 11). However, there was no significant difference in the mean increase in iohexol permeability between the asymptomatic ($0.19 \pm 0.18\%$) and the symptomatic ($0.16 \pm 0.15\%$) runners.

RESULTS

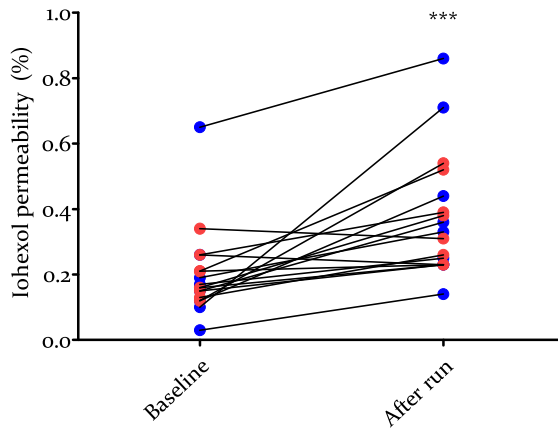


Figure 11. Running for 90 minutes significantly increased intestinal permeability to iohexol (% of administered iohexol) in both study groups. Blue dots represent individual values from the asymptomatic group and red dots from the symptomatic group. The lines connecting the dots indicate the change from the baseline in each individual. *** $P < 0.001$. $n = 9$ in the asymptomatic, $n = 8$ in the symptomatic.

5.3.3 DCA increases ex vivo permeability to fluorescein in mucosal segments

Subjecting jejunal mucosal tissues to DCA concentrations relating to high-fat feeding for 20 min significantly ($P < 0.001$) increased permeability to fluorescein ($26.5 \pm 3.2 \times 10^{-3}$ cm/h) compared to non-treated mucosal segments ($19.5 \pm 3.0 \times 10^{-3}$ cm/h) (Figure 12). In the intact tissues, DCA treatment did not increase fluorescein permeability (Figure 12) but resulted in a significant ($P < 0.001$) increase in tissue TER values ($17.5 \pm 11.8 \Omega/\text{cm}^2$) compared to spontaneous changes in TER values observed in the control tissues ($-1.4 \pm 3.2 \Omega/\text{cm}^2$) (Figure 13). Treating the intact tissues with TTX to eliminate neural activity prior to exposure to DCA significantly ($P < 0.001$) attenuated the DCA-induced increase in TER values ($4.8 \pm 9.3 \Omega/\text{cm}^2$).

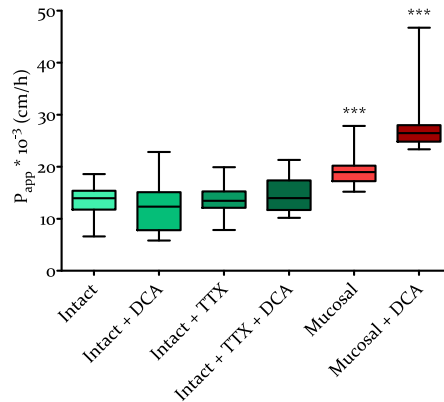


Figure 12. Treating jejunal tissue segments stripped of seromuscular layers (Mucosal) with 3 mM deoxycholic acid (DCA) for 20 minutes in Ussing chambers significantly increased tissue permeability to fluorescein. No changes in fluorescein permeability were observed in intact jejunal tissues treated with DCA or in intact tissues treated with 1 μM tetrodotoxin (TTX) prior to DCA-treatment. Box plots show median with upper and lower quartiles. Whiskers represent minimum and maximum. *** $P < 0.001$ compared to all other groups. $n = 9-22$.

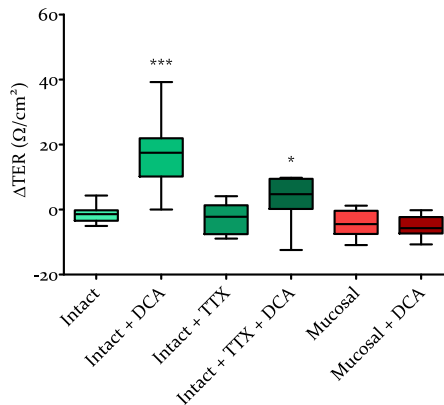


Figure 13. Exposing jejunal tissue segments to 3 mM deoxycholic acid (DCA) for 20 minutes in Ussing chambers resulted in a significant increase in transepithelial resistance (TER). Tissue segments treated with 1 μM tetrodotoxin (TTX) prior to DCA treatment exhibited a significantly attenuated response to DCA. Box plots show median with upper and lower quartiles. Whiskers represent minimum and maximum. * $P < 0.05$, *** $p < 0.001$ compared to all other groups. $n = 9-22$.

RESULTS

5.4 Analysis of fecal microbiota

5.4.1 Irinotecan modulates the composition of fecal microbiota

Irinotecan administration significantly ($P < 0.01$) decreased the diversity of fecal microbiota (calculated as inverse Simpson Index) compared to other treatment groups (Figure 14A). Inverse Simpson Index showed an inverse correlation with iohexol permeability (Spearman's $\rho = -0.412$, $P < 0.01$) (Figure 14B).

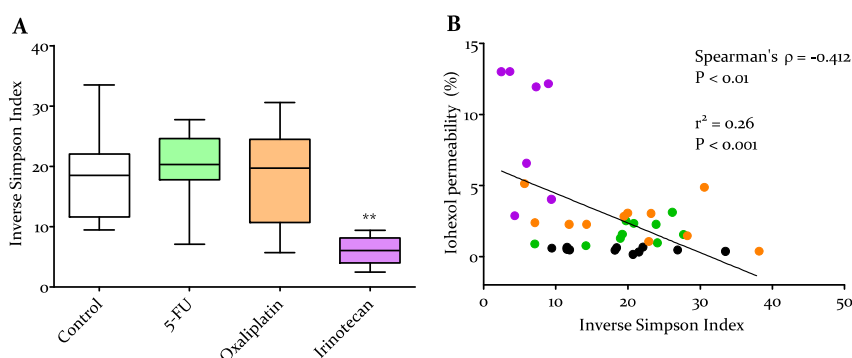


Figure 14. Irinotecan treatment resulted in a significant reduction in fecal microbial diversity (inverse Simpson Index) after 72 hours (A). The diversity of fecal microbiota at the end of the experiment showed an inverse correlation with intestinal permeability to iohexol (B). Black dots represent samples from the Control group, green dots from the 5-FU group, orange dots from the Oxaliplatin group, and purple dots from the Irinotecan group. Box plots show median with upper and lower quartiles. Whiskers represent minimum and maximum. ** $P < 0.01$ compared to all other groups. $n = 7-11$. Modified from two figures originally appearing in *Cancer Chemotherapy and Pharmacology* (80:317-332, 2017) and reprinted under the Creative Commons license.

At the phylum level, irinotecan significantly altered the composition of fecal microbiota by reducing the relative abundances of Actinobacteria ($P < 0.05$), Bacteroidetes ($P < 0.001$), and Synergistetes ($P < 0.001$) while increasing the relative proportions of Proteobacteria ($P < 0.01$) and Fusobacteria ($P < 0.001$)

compared to the Control group (Table 8). The genus level analysis revealed that the irinotecan-induced increase in the relative abundance of Proteobacteria was mostly driven by a significant ($P < 0.01$) increase in *Escherichia/Shigella* spp.

Table 8. Phylum level changes in the relative abundances (%) of each bacterial taxon

Phylum	Control	5-FU	Oxaliplatin	Irinotecan
Actinobacteria	$-5.9 \pm 6.0\%^a$	$0.01 \pm 3.3\%$	$-9.4 \pm 10.7\%$	$-8.0 \pm 9.5\%^a$
Bacteroidetes	$4.1 \pm 23.4\%^c$	$19.7 \pm 19.1\%^c$	$10.4 \pm 18.0\%^c$	$-16.4 \pm 22.0\%^{c,c,c}$
Firmicutes	$-1.9 \pm 28.6\%$	$-27.8 \pm 18.8\%$	$-19.7 \pm 25.8\%$	$-1.8 \pm 62.5\%$
Fusobacteria	$0.0 \pm 0.1\%^c$	$0.0 \pm 0.03\%^b$	$0.05 \pm 5.5\%$	$13.9 \pm 16.1\%^{b,c}$
Proteobacteria	$0.6 \pm 1.0\%^b$	$4.8 \pm 9.0\%$	$8.8 \pm 18.7\%$	$10.2 \pm 38.5\%^b$
Synergistetes	$0.4 \pm 0.8\%^c$	$0.55 \pm 0.85\%^b$	$0.15 \pm 1.2\%$	$-0.7 \pm 0.8\%^{b,c}$
Verrucomicrobia	$0.4 \pm 0.3\%^b$	$2.0 \pm 2.1\%^{a,b}$	$0.8 \pm 3.2\%$	$0.1 \pm 1.2\%^a$

Values are expressed as means \pm standard deviations. ^a $P < 0.05$ between groups, ^b $P < 0.01$ between groups, ^c $P < 0.001$ between groups. Modified from a table originally appearing in *Cancer Chemotherapy and Pharmacology* (80:317-332, 2017) and reprinted under the Creative Commons license.

RESULTS

5.5 Analysis of global metabolome

5.5.1 Chemotherapeutics change the global metabolic profile of serum and urine

PCA of serum and urine revealed that the chemotherapy groups were metabolically different from the Control group at the end of experiment (Figure 15). In the serum analysis, the control samples formed a separate cluster away from the baseline samples indicating also time-dependent changes in the metabolome. VIP scores derived from PLS-DA modeling of serum and urine metabolome indicated several metabolites that were relevant for the observed discrimination between the Control group and each treatment group (Figure 16).

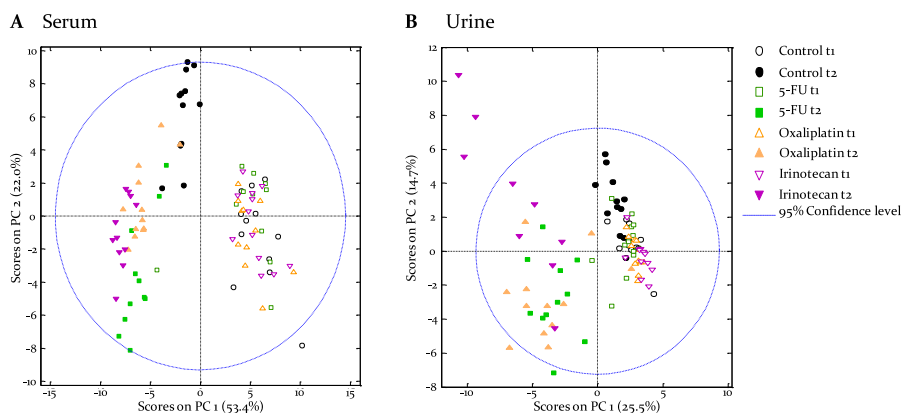


Figure 15. Principal component analysis (PCA) of the serum metabolome (A) showed both time-dependent and chemotherapeutics-induced changes at the end of the experiment (t₂, filled-out shapes). The urine metabolome (B) exhibited only chemotherapy-related alterations. At baseline (t₁, empty shapes), the groups clustered together in both analyses exhibiting metabolic similarity. PC = principal component. Modified from a figure originally appearing in *Cancer Chemotherapy and Pharmacology* (80:317-332, 2017) and reprinted under the Creative Commons license.

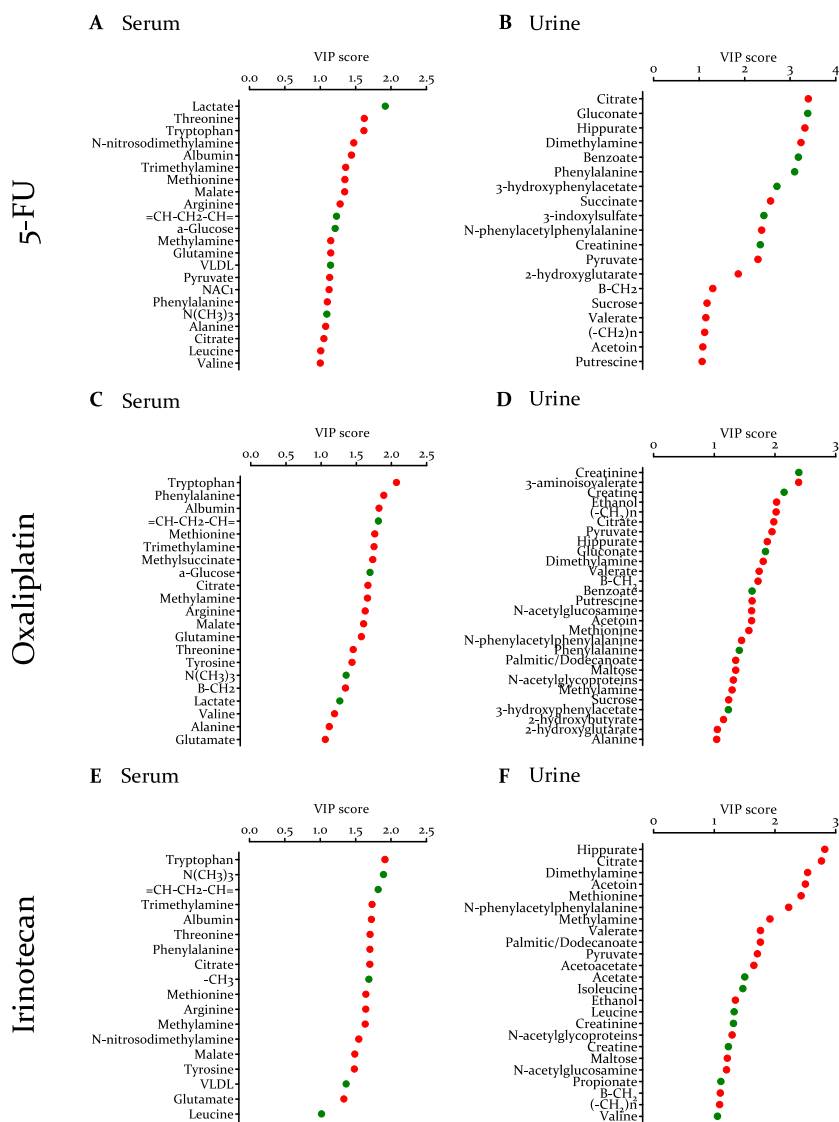


Figure 16. Variable importance in the projection (VIP) scores derived from partial least squares-discriminant analyses (PLS-DA) of serum (left panel: A, C, E) and urine (right panel: B, D, F). VIP scores indicate the individual metabolites that are the most relevant for discrimination between each treatment group (5-FU: A, B; Oxaliplatin: C, D; Irinotecan: E, F) and the Control group. The color of the dots depicts the direction of the metabolic change relative to the Control group (green dots: significant increase in metabolite's resonance intensity compared to the Control group; red dots: significant decrease in metabolite's resonance intensity compared to the Control group).

RESULTS

5.5.2 Chemotherapeutics increase serum levels of fatty acid and $N(\text{CH}_3)_3$ moieties

All the administered chemotherapeutics significantly increased the serum levels of fatty acid moieties $-\text{CH}_3$ ($P < 0.01$) and $=\text{CH}-\text{CH}_2-\text{CH}=$ ($P < 0.001$) (Figure 17). These moieties correspond to LDL-like lipid particles ($-\text{CH}_3$) and polyunsaturated fatty acids (PUFAs; $=\text{CH}-\text{CH}_2-\text{CH}=$). Similarly, the animals that received chemotherapeutics had significantly ($P < 0.001$) elevated levels of serum $N(\text{CH}_3)_3$ moieties compared to control animals (Figure 17C). This moiety is present in multiple trimethylamine- and choline-containing metabolites.

5.5.3 Chemotherapeutics decrease urinary hippurate excretion

The chemotherapy-treated groups excreted significantly ($P < 0.001$) less hippurate in urine than the Control group (Figure 18A). In addition, urinary hippurate excretion exhibited a significant inverse correlation with iohexol permeability (Spearman's $\rho = -0.887$, $P < 0.001$) (Figure 18B).

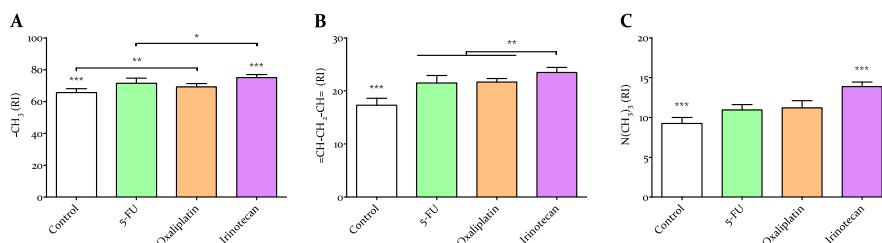


Figure 17. Chemotherapeutics significantly increased the serum levels of fatty acid moieties $-\text{CH}_3$ (A) and $=\text{CH}-\text{CH}_2-\text{CH}=$ (B) as well as the $\text{N}(\text{CH}_3)_3$ moiety (C). Bars show the mean resonance intensities (RI) with the error bars representing standard deviations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $n = 10-12$.

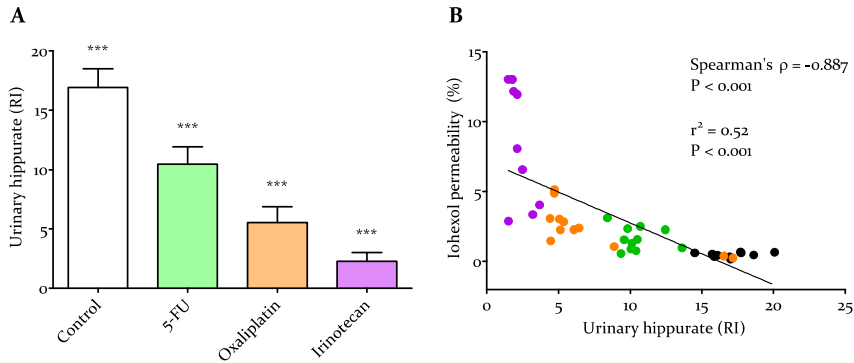


Figure 18. Chemotherapeutics significantly decreased urinary hippurate excretion (A). The excretion of hippurate also showed an inverse correlation with intestinal permeability to iohexol (% of administered iohexol) (B). Black dots represent samples from the Control group, green dots from the 5-FU group, orange dots from the Oxaliplatin group, and purple dots from the Irinotecan group. Bars show the mean resonance intensity (RI) with the error bars indicating standard deviations. *** $P < 0.001$ compared to all other groups. $n = 9-12$.

5.6 Biochemical analyses

5.6.1 Running increases serum concentrations of I-FABP

Overall, running for 90 minutes significantly ($P < 0.01$) increased serum I-FABP concentrations (Figure 19). However, there was no significant difference in the mean increase of serum I-FABP concentrations following the running test between asymptomatic (490 ± 513 pg/ml) and symptomatic (572 ± 832 pg/ml) runners.

5.6.2 Other biochemical measurements

Fecal calprotectin concentrations showed a non-significant ($P = 0.058$) increase from baseline after the 90-min running test (Figure 20).

Running for 90 minutes did not increase serum LPS activity but symptomatic runners exhibited significantly ($P < 0.01$) higher serum LPS activity at baseline

RESULTS

than their asymptomatic counterparts (Figure 21). After the running test, there was no difference in serum LPS activity between the groups.

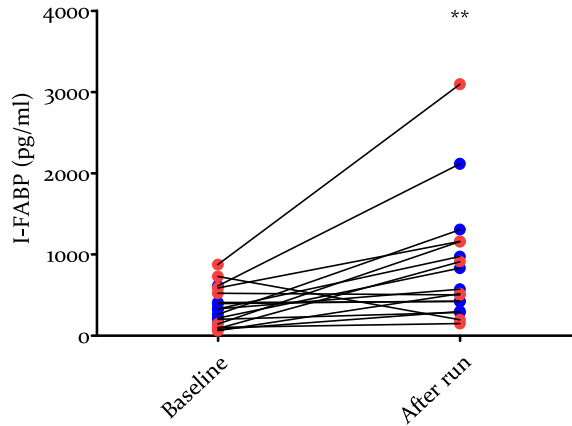


Figure 19. Running for 90 minutes significantly increased the serum concentration of intestinal fatty acid-binding protein (I-FABP). Blue dots represent individual values from the asymptomatic group and red dots from the symptomatic group. The lines connecting the dots indicate the change from the baseline in each individual. $**P < 0.01$. $n = 9$ in the asymptomatic, $n = 8$ in the symptomatic.

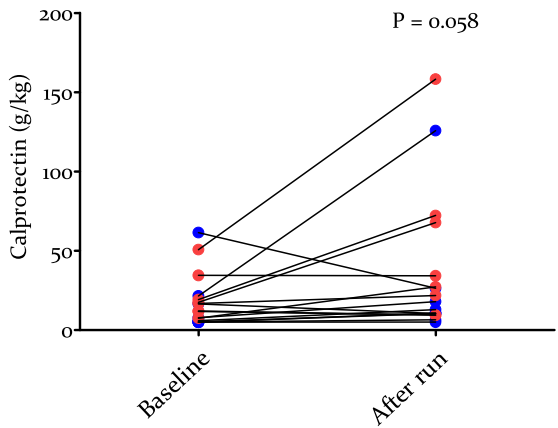


Figure 20. Fecal calprotectin concentration tended to increase after a 90-min running test. Blue dots represent individual values from the asymptomatic group and red dots from the symptomatic group. The lines connecting the dots indicate the change from the baseline in each individual. $n = 9$ in the asymptomatic, $n = 8$ in the symptomatic.

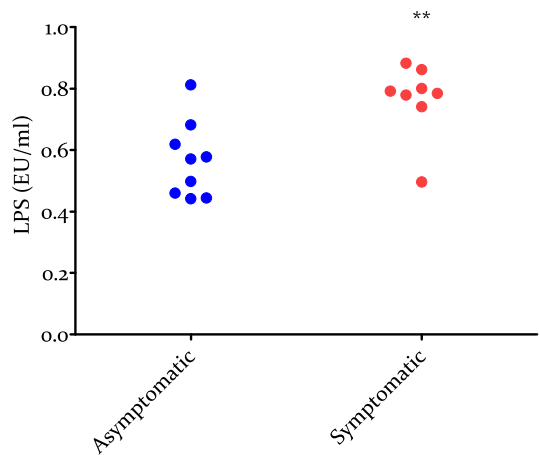


Figure 21. Athletes who reported to experience gastrointestinal symptoms over 50% of runs (symptomatic) had higher serum bacterial lipopolysaccharide (LPS) activity at rest than asymptomatic runners. After a 90-min running test, however, both groups exhibited similar serum LPS activity. $^{**}P < 0.01$.

6 DISCUSSION

6.1 Chemotherapy-induced gastrointestinal toxicity

6.1.1 Experimental models and study setting

Our results from Study I/II demonstrate the detrimental effects of chemotherapy to the intestinal mucosa as the experimental animals that received 5-FU, oxaliplatin, or irinotecan exhibited significant body weight loss, diarrhea, and intestinal damage. These findings are consistent with other animal studies in which body weight loss, varying degrees of diarrhea, and histopathological changes in the intestine are commonly observed following the administration of 5-FU, oxaliplatin, or irinotecan (Generoso et al., 2015; Justino et al., 2014; Kato et al., 2017; Lima-Júnior et al., 2014; Vanhoecke et al., 2015; Wang et al., 2015). In clinical studies, gastrointestinal toxicity is usually reported as diarrhea incidence and severity, both of which vary greatly depending on the chemotherapy-regimen (Andreyev et al., 2014). Across different studies, the average incidence of severe diarrhea during chemotherapy seems to be approximately 15% (Andreyev et al., 2014; Iacovelli et al., 2014). Interestingly, the risk of severe diarrhea during chemotherapy increases with treatments containing irinotecan suggesting that irinotecan is particularly damaging to the intestine (Andreyev et al., 2014; Iacovelli et al., 2014; Keefe et al., 2014). In our study, irinotecan treatment resulted in the most severe form of diarrhea and significantly greater acute colonic damage. These two events are likely related because fluid adsorption happens mainly in the large intestine. Also, mechanistically, irinotecan's propensity to damage the colon may explain the increased incidence of severe diarrhea observed with irinotecan-based regimens.

Although our experimental animals exhibited clear signs of CIGT, it is worth considering the clinical relevance of the administered drug doses. We employed a single injection model for all chemotherapeutics with doses designed to induce gastrointestinal toxicity but not mortality. Our drug administration

routes and dose selection was based on previous literature describing the gastrointestinal effects of different doses of 5-FU (Cool et al., 2005), oxaliplatin (Wang et al., 2015), and irinotecan (Gibson et al., 2007) in rodents. How these models compare to the clinical situation is difficult to decipher. Firstly, in humans, chemotherapy-regimens usually consist of a combination of drugs which are administered intravenously in several cycles over multiple weeks. Secondly, human chemotherapy doses are calculated as milligrams per body surface area. For example, a commonly used FOLFIRI regimen includes an intravenous bolus of 400 mg/m² and a 46-h intravenous infusion of 2400 mg/m² 5-FU as well as an intravenous infusion of 180 mg/m² irinotecan over 90 minutes. In oxaliplatin-containing regimens such as FOLFOX, the dosage of oxaliplatin is usually 85 mg/m² (personal communication with P. Österlund). Estimation on how these doses compare to the doses in our study can be done using the following equation (Nair and Jacob, 2016):

Human equivalent dose (HED)

$$= \text{Animal dose mg/kg} \times \left(\frac{\text{Animal weight (kg)}}{\text{Human weight (kg)}} \right)^{0.33}$$

Using the average body weight of animals in our study (0,332 kg) and a reference human body weight of 60 kg, the HEDs of our doses were 27 mg/kg for 5-FU, 2.7 mg/kg for oxaliplatin, and 36 mg/kg for irinotecan. These values can further be converted to per body surface area values by multiplying them by a correction factor of 37 (Nair and Jacob, 2016). This yields the final HED values of 1000 mg/m² for 5-FU, 100 mg/m² for oxaliplatin, and 1330 mg/m² for irinotecan. These values reveal that our doses for 5-FU and oxaliplatin somewhat represent the doses used in common chemotherapy-regimens whereas the irinotecan dose was clearly higher than what is normally administered to patients. However, considering that pharmacokinetic properties of drugs usually differ between species, scaling and directly comparing drug doses between humans and rodents should be interpreted carefully.

DISCUSSION

6.1.2 Does iohexol permeability reflect the severity of CIGT?

Our data show that 5-FU, oxaliplatin, and irinotecan increase intestinal permeability to iohexol and that iohexol permeability correlates with the clinical manifestations of CIGT (body weight loss and diarrhea) and with the extent of acute colonic injury. Studies have previously reported increased intestinal permeability after the administration of 5-FU (Ferreira et al., 2012; Generoso et al., 2015; Justino et al., 2014) and irinotecan (Pedroso et al., 2015; Wardill et al., 2016) but, to our knowledge, our study is the first one to show increased intestinal permeability following oxaliplatin administration. Overall, these data suggest that iohexol permeability could serve as an objective measure of CIGT in clinical settings. This idea lends support from previous studies that have correlated increases in intestinal permeability after chemotherapy with the severity of mucositis (Choi et al., 2007; Melichar et al., 2007) and the occurrence of diarrhea (Russo et al., 2013) and nausea (Fazeny-Dörner et al., 2002). Melichar et al. (2007) even showed that pre-chemotherapy intestinal permeability measurements could possibly predict individual patients' susceptibility to CIGT. Thus, measuring intestinal permeability prior to or during chemotherapy could help clinicians to adjust dosing individually before the onset of severe toxicities. In this regard, iohexol's main advantage over other permeability probes would be that it is already readily available in medical facilities due to its routine use as a contrast medium. Iohexol is also easier to detect than the commonly used sugar probes that require chromatographic equipment for detection. However, considering that the above-mentioned studies employed either the L/M -ratio or ^{51}Cr -EDTA as their intestinal permeability probe, the use of iohexol permeability in the context of CIGT warrants more research.

6.1.3 What do the microbial and metabolic changes reveal about the mechanisms of CIGT?

Recent studies have highlighted the role of microbiota in chemotherapy-related toxicities as chemotherapeutics not only modulate the composition of intestinal

microbiota (Touchefeu et al., 2014) but they are also subject to modulation by the intestinal microbes themselves (Alexander et al., 2017). Our microbial analysis show significant changes in the composition of fecal microbiota following the administration of irinotecan. Notably, irinotecan treatment reduced the diversity of fecal microbiota and increased the relative abundance of Proteobacteria with both events indicating intestinal dysbiosis, a condition often associated with intestinal inflammation (Shin et al., 2015; Suchodolski, 2016). All chemotherapeutics also exhibited significantly decreased urinary hippurate excretion which has been associated with intestinal dysbiosis in IBD patients (Williams et al., 2010). Fecal microbiota diversity and urinary hippurate excretion also correlated with intestinal permeability suggesting that intestinal dysbiosis may contribute to the development of CIGT.

Generally, our microbial data seem to be consistent with other animal studies examining irinotecan-induced modifications in fecal microbiota (Lin et al., 2012; Stringer et al., 2007; Touchefeu et al., 2014; Vanhoecke et al., 2015). Although one should be cautious when comparing changes in gut microbiota between species, Proteobacteria seem to be universally elevated in different species during intestinal inflammation (Larrosa et al., 2009; Lupp et al., 2007; Minamoto et al., 2015; Shin et al., 2015). Additionally, human studies with varying chemotherapy-regimens have also reported reduced microbial diversity and increased abundance of Proteobacteria following chemotherapy (Alexander et al., 2017; Montassier et al., 2015, 2014; Stringer et al., 2013). These are interesting findings in the context of CIGT because Proteobacteria produce LPS which together with the observed increase in intestinal permeability could lead to increased LPS leakage into circulation and subsequent activation of inflammatory processes. Indeed, studies have shown increased concentrations of blood LPS and pro-inflammatory cytokines after chemotherapy (Cario, 2016; Lee et al., 2014; Wardill et al., 2016). Interestingly, improper function of TLR4, the major receptor for LPS, protects mice from methotrexate-induced gut toxicity (de Koning et al., 2006) and the deletion of the TLR4 gene altogether decreases irinotecan-induced gastrointestinal damage (Wardill et al., 2016).

DISCUSSION

Although only irinotecan administration resulted in a statistically significant increase in the relative abundance of Proteobacteria, our metabolic analyses hint the presence of inflammatory processes in all chemotherapy-treated groups. All treatment groups exhibited increased levels of serum fatty acid and $\text{N}(\text{CH}_3)_3$ moieties which may be signs of inflammation-mediated lipolysis and PUFA generation. Increased plasma concentrations of PUFAs and $\text{N}(\text{CH}_3)_3$ moieties are also present in $\text{IL-10}^{-/-}$ mice which spontaneously develop colitis (Martin et al., 2009) suggesting that their increase is due to intestinal inflammation. Interestingly, in a previous clinical study, elevated pre-treatment levels of $-\text{CH}_3$, $=\text{CH}-\text{CH}_2-\text{CH}=\text{}$, and $\text{N}(\text{CH}_3)_3$ moieties associated with increased incidence of severe toxicities during capecitabine (5-FU pro-drug) treatment leading the authors to speculate about underlying inflammation in these patients (Backshall et al., 2011). In addition to serum lipids, all chemotherapy-treated groups in our study exhibited significantly decreased serum levels of several amino acids, such as tryptophan and arginine which have been shown to participate in inflammatory processes (Gupta et al., 2012; Popovic et al., 2007). Overall, these findings indicate that the relationship between intestinal microbiota, intestinal permeability, and inflammatory signals together influence the development of CIGT but the exact causality of this relationship is still unclear.

However, some limitations should be kept in mind when interpreting our results. Some of the observed changes may rise from catabolic effects of stress and poor nutritional status. The metabolic analyses revealed many metabolic alterations that could be related to the stressful experimental conditions, such as metabolic caging. Considering that changes in body weight may impact the levels of multiple metabolites, it is likely that some of our observed changes in the metabolome represent non-specific toxicities to the chemotherapeutics (Connor et al., 2004; Serrano-Contreras et al., 2016). Nonetheless, as the baseline samples and samples from control animals were also collected under/after metabolic caging, the alterations in the metabolome can, at least partly, be attributed to the chemotherapeutics.

6.2 Running-induced gastrointestinal distress

6.2.1 Does intestinal permeability explain gastrointestinal symptoms during running?

Our results from Study III show that running for 90 min at a challenging pace increases intestinal permeability to iohexol. Previous studies on this subject have reported mixed findings (Davison et al., 2016; JanssenDuijghuijsen et al., 2017; March et al., 2017; Marchbank et al., 2011; Pals et al., 1997; Ryan et al., 1996; van Nieuwenhoven et al., 2004, 1999; van Wijck et al., 2012b; van Wijck et al., 2011; Zuhl et al., 2015) but to our knowledge, our study is the first to directly compare running-induced changes in intestinal permeability in asymptomatic and symptomatic athletes. Our data suggest that changes in intestinal permeability do not explain the development of gastrointestinal symptoms during running as we observed no differences between asymptomatic and symptomatic runners. However, there are several factors that should be considered when interpreting these results.

Firstly, although we instructed the athletes to run at a challenging pace, we did not control their pace at any point during the running test. Our study design is similar to studies conducted during running competitions which might have led to differences in individual running intensities and affected their likelihood of developing gastrointestinal symptoms. Differences in running intensity could explain why the two groups experienced gastrointestinal symptoms at a similar rate after the running test. Our questionnaires were also mainly aimed at probing for symptom occurrence and not severity which could have masked some differences between the two groups. Overall, these factors emphasize the individual nature of gastrointestinal symptoms and the difficulty of accurately assigning individuals as asymptomatic or symptomatic.

DISCUSSION

6.2.2 Is intestinal ischemia the cause of increased intestinal permeability after running?

Exercise reduces intestinal blood flow which may result in intestinal ischemia and subsequent damage to the intestinal mucosa (van Wijck et al., 2012a). Our data show elevated serum I-FABP concentrations after the running test which could be interpreted as a sign of small intestinal ischemia and enterocyte damage (Adriaanse et al., 2013; Hundscheid et al., 2015; van Wijck et al., 2011). Whether small intestinal ischemia is the cause of exercise-induced increase in intestinal permeability is an interesting question. A total of two studies have previously reported concomitant increases in plasma I-FABP levels and small intestinal permeability following exercise (March et al., 2017; van Wijck et al., 2011) but contradictory findings also exist (van Wijck et al., 2014). Interpreting our results, it is essential to keep in mind that we employed a continuous 24-h intestinal permeability measurement and thus cannot distinguish between small intestinal and colonic permeability. Thus, our intestinal permeability values may not be directly comparable to the I-FABP values that represent mainly small intestinal damage. However, considering that the absorption of iohexol seems to be constant along the intestinal tract (Halme et al., 2000) and that comparable studies have used intestinal permeability measurement times ranging from 1 to 5 hours (Davison et al., 2016; JanssenDuijghuijsen et al., 2017; March et al., 2017; Marchbank et al., 2011; Pals et al., 1997; van Wijck et al., 2011; Zuhl et al., 2015), it is likely that the observed increase in intestinal permeability to iohexol in our study is a result of ischemia-induced damage to the small intestine.

6.2.3 Does serum LPS activity contribute to gastrointestinal symptoms during exercise?

Our data show that the study subjects classified as symptomatic had significantly higher serum LPS activity at rest than the asymptomatic subjects. In athletes, training-induced adaptations can impact resting blood LPS levels (Bosenberg et al., 1988; Lim et al., 2009) so it is possible that symptomatic

athletes lack adequate adaptation mechanisms and thus have chronically elevated LPS activity. However, whether LPS contributes to gastrointestinal symptoms during exercise is still under debate. Brock-Utne et al. (1988) showed that ultramarathon runners with high plasma LPS concentrations after running experienced more gastrointestinal symptoms than those with normal plasma LPS levels but others have not been able to show a similar correlation (Gill et al., 2015; Jeukendrup et al., 2000; Moncada-Jiménez et al., 2009). Hence, the relationship between LPS and gastrointestinal symptoms during exercise warrants more robust research that should considerate the individual differences in adaptations to LPS accumulation and clearance.

Additionally, although multiple studies have reported increased blood LPS concentrations after exercise (Ashton et al., 2003; Bosenberg et al., 1988; Brock-Utne et al., 1988; Gill et al., 2015; Jeukendrup et al., 2000; Lim et al., 2009; Moncada-Jiménez et al., 2009), these studies have not linked this to exercise-induced changes in intestinal permeability. In our study, despite increased intestinal permeability after running, we did not observe any significant increases in serum LPS activity after the running test. This finding is similar to the only other study that has measured blood LPS concentrations simultaneously with a permeability marker (urinary claudin-3 concentration) (Yeh et al., 2013). In theory, increased intestinal permeability and LPS leakage into circulation could activate inflammatory processes. Our analysis show that running increased fecal calprotectin concentration with symptomatic athletes exhibiting on average twice as high values as the asymptomatic runners. Albeit this increase did not reach statistical significance, it indicates the activation of inflammatory processes in the intestinal mucosa and supports the role of inflammation in the development of gastrointestinal symptoms during exercise (Gill et al., 2015; Jeukendrup et al., 2000).

DISCUSSION

6.3 DCA-induced increase in macromolecular permeability

6.3.1 Does DCA increase macromolecular permeability via activation of enteric nerves?

High-fat diet increases the concentration of bile acids in the intestinal lumen and it has been associated with increased intestinal permeability. Multiple studies have previously shown the detrimental effects of hydrophobic bile acid DCA on the intestinal barrier function (Chadwick et al., 1979; Fihn et al., 2003; Goerg et al., 1980, 1983; Hughes et al., 2008; Münch et al., 2007; Stenman et al., 2013a; Sun et al., 2004) with a few studies reporting neural mechanisms behind this effect (Fihn et al., 2003; Sun et al., 2004). Our data show that DCA can increase macromolecular permeability independent of functional enteric nerves suggesting the presence of other mechanisms, such as tissue damage (Stenman et al., 2013a) or tight junctional modifications (Raimondi et al., 2008). However, treating the intestinal tissues with TTX to eliminate neural activity prior to DCA treatment stunted the DCA-induced increase in tissue TER values. This indicates the involvement of some neural component. Overall, the observed increase in TER values is interesting because it contrasts with our previous findings (Stenman et al., 2013a). A possible explanation for it could be DCA-mediated chloride secretion via cystic fibrosis transmembrane conductance regulator (CFTR) which collapses the lateral intercellular space (LIS) between adjacent enterocytes resulting in increased TER values and subsequently decreased paracellular permeability (Gawenis et al., 2004). This sequence may include a neural pathway that involves DCA-induced serotonin secretion (Peregrin et al., 1999; Rolfe and Levin, 1998). Nonetheless, we observed no changes in macromolecular permeability after DCA treatment in any of the intact tissues. Because we conducted this experiment in Ussing chambers, it is possible that the seromuscular layer presented a physical barrier that prevented us from seeing DCA-induced changes in permeability. This highlights some of the limitations associated with this method. However, considering that we employed a DCA concentration corresponding to that observed in fecal matter

DISCUSSION

of mice during high-fat feeding (Stenman et al., 2012), these findings corroborate the emerging hypothesis that hydrophobic bile acids may contribute to the barrier-disrupting effects of high-fat diet.

7 CONCLUSIONS

This thesis consists of three studies where we investigated changes in intestinal permeability after different treatment protocols. The main findings area as follows:

- I. Commonly used chemotherapeutics, 5-FU, oxaliplatin, and irinotecan increase intestinal permeability to iohexol and this increase correlates with the clinical features of CIGT and with colonic damage. This study is also the first one to show that oxaliplatin increases intestinal permeability.
- II. Intestinal permeability to iohexol shows capability to act as a simple tool for objectively assessing the severity and occurrence of CIGT in a clinical setting.
- III. The pathophysiology of CIGT is associated with several changes in the composition of intestinal microbiota and in the metabolome. The observed changes suggest that intestinal dysbiosis together with increased intestinal permeability leads to the activation of inflammatory processes.
- IV. Running increases intestinal permeability to iohexol via mechanisms that involve intestinal ischemia but this increase does not explain the occurrence of gastrointestinal symptoms in individual athletes.
- V. A DCA concentration associated with high-fat feeding increases macromolecular permeability to fluorescein in murine small intestine. Although DCA elicited also a partial neural response, the increase in macromolecular permeability seems to be mediated by direct interaction with the mucosa and does not involve neural mechanisms.

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